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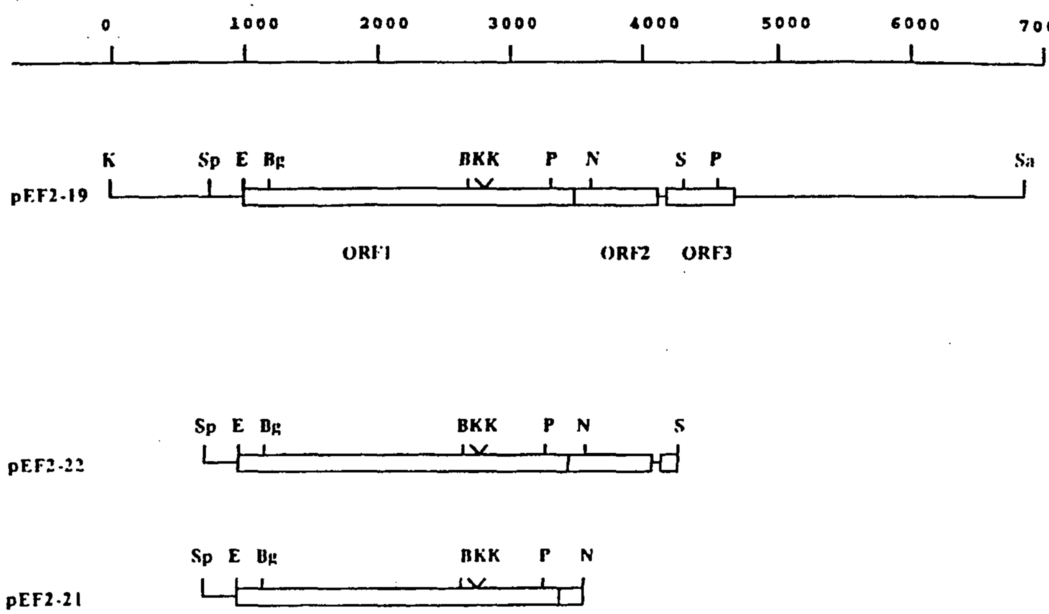
(54) Title: DNA SEQUENCES WHICH CODE FOR VIRULENCE CHARACTERISTICS OF *STREPTOCOCCUS SUIS* AND PARTS THEREOF, POLYPEPTIDES AND ANTIBODIES DERIVED THEREFROM AND THE USE THEREOF FOR THE DIAGNOSIS OF AND PROTECTION AGAINST INFECTION BY *S. SUIS* IN MAMMALS, INCLUDING MAN



(57) Abstract

The invention provides DNA sequences which code for polypeptides which are characteristic for the virulence of the pathogenic bacterium *Streptococcus suis* and parts thereof, and polypeptides and antibodies derived therefrom. The sequences code for a polypeptide of 90.000-120.000 daltons or a polypeptide of higher molecular weight containing such a polypeptide, and for a polypeptide of 135.000-136.000 daltons (muramidase released protein), or parts thereof. The sequences themselves, and also the polypeptides and antibodies derived therefrom, are used for diagnosis of and protection against infection by *S. suis* in mammals, including man.

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DNA sequenc s which code for virulence characteristics of *Streptococcus suis* and parts thereof, polypeptides and antibodies derived therefrom and the use thereof for the diagnosis of and protection against infection by *S. suis* in mammals, including man.

The invention is in the field of veterinary and human preventive medicine, in particular that of the diagnosis of and protection against infection by pathogenic strains of the bacterium *Streptococcus suis*.

Infections with *Streptococcus suis* serotype 2 in young pigs at about the time of weaning have been a growing problem in the Netherlands since 1983. The disease is characterised by meningitis, arthritis, sepsis and death (Clifton-Hadley 1983, *ref. 6*; Vecht et al. 1985, *ref. 44*; Windsor 1977, *ref. 50*). It is estimated that 5-10 per cent of farms have problems of this type. The mortality is estimated at 2.5% and the morbidity in affected farms is on average 2-5%. Therapeutic and preventive measures have only a limited effect. The economic damage is accordingly appreciable. The disease is a zoonosis. Humans are also susceptible to this infection, with the risk of sepsis and meningitis with possibly permanent side-effects; rare cases of death have been reported (Arends and Zanen 1988, *ref. 2*). This related mostly to cases of people with a skin wound coming into contact with infected pork. In particular, pig farmers and slaughterhouse staff belong to the risk group.

There are indications that the increased rate of illness on pig farms in the Netherlands since 1983 is to be ascribed to the import of breeding animals which are carriers of *S. suis* type 2. Carriers are often healthy adult pigs which harbour the streptococci in the tonsils and mucosa of the upper respiratory tract. The infection is transmitted via these carriers to susceptible animals, frequently piglets at weaning age. Diagnosis of animals which are already sick or have died is based on isolation and determination of *S. suis* type 2 from clinical samples or organs after necropsy. Detection of carriers is based on bacteriological examination of nose or throat swabs or tonsil biopsies using a selective/elective medium (Van Leengoed et al. 1987, *ref. 27*). On the basis of diagnostic testing to detect carriers, it should be possible to set up a control programme. However, testing for carriers using the conventional becteriological techniques is time-consuming, which complicates the processing of large numbers of samples; there is also a risk of false negative results due to overgrowth with contaminants.

Finally, interpretation of the t st demands a great deal of experience. Moreover, diagnosis and possible control on the basis of diagnosis are further complicated by the occurrence of differences in pathogenicity within the *S. suis* type 2 species. Regular testing for carriers within a control programme is sensible only if truly virulent strains of *S. suis* type 2 can be differentiated from avirulent strains. Current diagnostic techniques do not make such discrimination. Consequently, control based on the detection of carriers of virulent *S. suis* type 2 strains is not yet possible.

Differences in virulence are ascribed, inter alia, to the presence or absence of virulence factors. In 1984, Arends and Zanen (*ref. 1*) already described "lysozyme-positive proteins" in human strains. In a study with experimental animals it was found that a "lysozyme-positive" strain (D-282) was pathogenic for gnotobiotic pigs, in contrast to a "lysozyme-negative" strain (T-15) (Vecht et al. 1989, *ref. 43*). The "lysozyme-positive protein" is probably identical to the muramidase-released protein (MRP) of strain D-282.

The pig industry in the Netherlands and many other countries has a pyramid structure, with a small number of breeding herds at the top, from where animals are distributed to multiplication herds. These supply a large number of fattening herds, from where the (animal) product finally goes to the slaughterhouses. A control program based on diagnosis (certification of farms, elimination of positive carriers, import requirements) should primarily aim at creating herds which are free of *S. suis* type 2 high in this pyramid. A vaccine would primarily be useful in affecting herds lower in the pyramid. Furthermore, means and methods for diagnosing infections by *Streptococcus suis* in human medicine can be of value.

The object of the invention is to provide methods and means which make it possible, in a more effective manner than hitherto, to detect infections by *Streptococcus suis* on the one hand and to prevent such infections by elimination of infected and carrier pigs on the other hand.

This object is achieved by using a DNA sequence from the gene which codes for a virulence characteristic of *S. suis*. In this context, a virulence characteristic is defined as a polypeptide whose presence is associated with the virulence of an organism, in this case the bacterium *S. suis*, in particular serotype 2.

Two genes of virulent strains of *S. suis* type 2 have been found which code for two proteins, which are designated MRP (muramidase

r leased protein) and EF (extracellular factor) and which appear to be characteristic for virulence (virulence factors). MRP and EF are high molecular weight proteins. MRP (136 kD) is a protein associated with the cell envelope and can be released from the cell wall by muramidase. EF (110 kD) is an extracellular product which is secreted by the bacterium into the growth medium. EF has higher molecular weight counterparts which are denoted herein as EF*.

The invention provides new diagnostic methods which are able to differentiate between virulent and avirulent strains. These methods are based on the genes encoding MRP, EF and EF* and their expression products. On the basis of the expression of one or both proteins by said genes, three different phenotypes of *S. suis* type 2 have been found to date: i.e. the MRP+ EF+ phenotype, the MRP+ EF- phenotype and the MRP- EF- phenotype. 77% (n = 111) of strains isolated from organs of pigs showing clinical symptoms of disease were found to possess the MRP+ EF+ phenotype, while 86% (n = 42) of isolates from tonsils of non-suspect normal slaughter pigs were found to possess the MRP- EF- phenotype. The MRP+ EF- phenotype was most frequently found (74%) (n = 27) in isolates from human patients with infections of *S. suis* type 2 (see Figure 10). Hence infected animals and carriers of virulent strains can be detected, and a vaccine based on MRP, EF and/or EF* can be developed. It is thus possible to detect carriers of virulent strains of *S. suis* and a vaccine can be developed. Using the diagnostic methods for detecting carriers and infected pig herds and/or using vaccines based on MRP, EF and/or EF*, a program for controlling infections by *S. suis* type 2 in pig herds can be developed.

The invention therefore relates to the DNA sequence of the gene which, apart from coding for specific high molecular weight polypeptides, codes for the 90-120 kDa polypeptide which is a characteristic of *S. suis* virulence, which gene, hereinafter designated the *ef* gene has the nucleotide sequence according to Fig. 1A for *S. suis* serotype 2, strain D-282, and to equivalent sequences and to parts of said sequences. The nucleotide sequence of the entire region coding for EF and the flanking sequences have been determined. Analysis of the sequence of the *ef* gene (Fig. 1A) provides an open reading frame of 2529 nucleotides which codes for a polypeptide of 843 amino acids (calculated molecular weight 90,014). Monoclonal antibodies generated against the 110 kDa EF protein recognised proteins with a higher molecular weight in culture supernatants of all 38 strains with a MRP+ EF- phenotype. This indicates that

certain epitopes of the 110 kDa EF and the high molecular weight proteins are identical. None of the 91 strains with a MRP+ EF+ phenotype was found to produce these high molecular weight proteins. At the same time, DNA probes based on the gene which codes for the 110 kDa EF were found to hybridise with genes which code for the high molecular weight proteins of MRP+ EF- strains. This indicates that the 110 kDa EF and the high molecular weight proteins are related, which implies that at least part of the *ef* gene, from strains with a MRP+ EF- phenotype, is identical to the *ef* gene of strains with the MRP+ EF+ phenotype. The higher molecular weight counterpart of the protein EF is designated herein as EF*, and the gene encoding it as the *ef** gene. The corresponding nucleotide and amino acid sequences are represented in Fig. 1B.

The invention also relates to the DNA sequence of the gene which codes for the 135-136 kDa polypeptide which is also a virulence characteristic of *S. suis*, which gene, hereinafter designated the *mrp* gene, has the nucleotide sequence according to Figure 2 for *S. suis* serotype 2 strain D-282, and to equivalent sequences and to parts of said sequences. The nucleotide sequence of the entire region coding for MRP and the flanking sequences have been determined. Analysis of the sequence of the *mrp* gene (Fig. 2) shows an open reading frame of 3768 nucleotides which codes for a polypeptide of 1256 amino acids (calculated molecular weight 135.794).

In this context, an equivalent sequence comprises a sequence which is essentially the same as the sequence shown but can display slight differences, such as point mutations, or other modifications which may be caused by substitution, deletion, insertion or addition; similarly, an equivalent sequence also comprises a sequence which, despite any differences in nucleotide sequence, hybridises with the sequence shown or with its complement, and also a related sequence which means that it codes for the same amino acid sequence despite differences in nucleotide sequence.

The invention also relates to a recombinant polynucleotide which contains an *ef/ef** gene and/or *mrp* gene sequence as described above, in the presence of a regulating sequence. A recombinant of this type, such as a virus vector, a plasmid or a bacterium, can be used for expression of the gene or of relevant parts thereof in a desired environment, for example for the production of immunogenic peptides intended for the diagnosis of an infection, or for controlling infections with virulent strains of *S. suis* by vaccination.

Polynucleotide prob s which contain a sequence as described above, derived from a gene which codes for a virulence characteristic of *S. suis*, also form part of the invention. A probe of this type in particular corresponds with part of the nucleotide sequence f one of the two said genes. The probe can be used for direct detection of the presence of sequences of virulent strains of *S. suis*. The probe can also be used as a basis for a primer for the multiplication of polynucleotides (for example in a polymerase chain reaction) as part of a diagnostic method or a protection method.

A suitable polynucleotide probe was found to be a partial sequence containing at least 10 nucleotides, preferably at least 15 nucleotides, up to 835 nucleotides from the sequence 1100-1934 of the *mrp* gene. Another suitable polynucleotide probe was found to be a partial sequence containing 10-417, in particular 15-417 nucleotides from the sequence 2890-3306 of the *ef** gene. These probes differentiate effectively between pathogenic and non-pathogenic strains of *S. suis*. A combination of such an *mrp* based probe and an *ef** based probe is an especially powerful diagnostic tool.

The invention also relates to polypeptides which are derived from a polynucleotide sequence described above. A polypeptide of this type is either coded by said sequence or obtained by expression of said sequence and essentially corresponds to a *S. suis* protein characteristic of virulence, or to a part thereof. A polypeptide of this type can, for example, be used as an antigen in an immunoassay, as an immunogen in the immunisation of mammals or as an immunogen for the production of antibodies for diagnostic purposes. The antibodies generated in this way also form part of the invention. Such antibodies can be polyclonal or monoclonal and can be conjugated with a marker (enzyme, isotope, luminescent substance or complex-forming agent); the antibody can also be bound to solid carriers.

The invention also relates to methods for the detection of an infection by a pathogenic strain or by a non-pathogenic strain of *S. suis*, in which one or more polynucleotide probes, polypeptides and/or antibodies as described above are used. "Infection" signifies here the presence of the pathogenic organism, both in the case where there are clinical signs of disease (infection in a narrow sense) and in the case where there are no clinical signs of disease (infection in a broad sense, or contamination). For immunoassays, such as a det rmination of the presence of antigens of and/or antibodies against *S. suis* in a sample or

in clinical material, it is possible, for xample, to use on a microtiter plate a polypeptide (110 kDa) which is encoded by the *ef/ef** gene or a part thereof, and/or an antibody which has been generated against such a polypeptide. In addition, it is also possible to use a polypeptide (136 kDa) encoded by the *mrp* gene or a part thereof, and/or an antibody which has been generated against such a polypeptide. The diagnostic methods can be carried out using procedures known per se. Examples are Enzyme-Linked Immunosorbent Assays (ELISA) and Double Antibody Sandwich (DAS)-ELISA.

The methods described above can be carried out with the aid of diagnostic kits. A diagnostic kit according to the invention contains, respectively, at least one polynucleotide or a polypeptide which corresponds to or is derived from a sequence of the *ef/ef** gene or *mrp* gene or a part thereof or contains an antibody which has been generated against the polypeptide derived from one of the said *ef/ef** and *mrp* sequences. It is also possible to use combinations of probes and the like, in particular of *ef** diagnostic agents and *mrp* diagnostic agents, or combinations of primers, for example for carrying out PCR. The kits can also contain the components required for carrying out diagnoses, such as reagents (labelling substances, dyes and the like), supports (filters, plates and the like), media and calibrating agents as well as a manual for carrying out the diagnosis.

The invention also relates to a method for protecting mammals against infection with *Streptococcus suis*, in which method a polynucleotide, a polypeptide or an antibody as described above is used. When an antibody is used, the method is a passive immunisation, that is to say there is direct provision of antibodies against the pathogenic organism; since antibodies which are derived from EF, EF* and MRP are directed against the most virulent forms of *S. suis*, a procedure of this type can be an effective method for protecting against, or controlling, infection, especially if the animal to be protected is not itself able to produce sufficient antibodies, for example if infection has already taken place or in the case of young animals.

Another form of passive immunisation in the case of pigs is the administration of antibodies to the piglets via the colostrum from the sow. In this case the dam is actively immunised with one or both polypeptides during pregnancy, that is to say before the birth of the piglets. When a polypeptide or a polynucleotide (optionally in the form of a recombinant organism) is used, the procedure is an active immunisa-

tion, the animal to be protected being stimulated, by means of the immunogenic polypeptide which is administered directly or in the form of a gene for expression, to produce antibodies.

Another suitable method of immunisation is the administration of a polypeptide from which the activity responsible for virulence has been neutralised. Such a polypeptide should then no longer be pathogenic, while immunogenic characteristics are retained. It can be obtained, for example, by expression of a gene which has been modified with respect to the original *ef/ef** or *mrp* gene, such as by means of deletion.

Vaccines for protecting mammals against an infection by *S. suis*, which vaccines contain a polynucleotide, a polypeptide or an antibody as described above, also form part of the invention.

A particular vaccine according to the invention is a vaccine which contains a *S. suis* material which does not or does not completely bring to expression at least one of the polypeptides corresponding to EF and MRP. This material can originate from or can be formed by a possible live strain which is not virulent or is less virulent.

The role of virulence factors which are involved in the pathogenesis of *S. suis* type 2 has been studied *in vivo* by means of gnotobiotic/germ-free piglets with *S. suis* type 2 strains defined in respect of virulence factors (MRP and EF). The animal experiments were monitored by means of haematological, bacteriological and (histo)-pathological analytical techniques.

Description of the figures

Figure 1A:

Nucleotide sequence of the *ef* gene and the adjacent sequences and the EF amino acid sequence derived therefrom. The presumed ribosome binding site, the -35 and -10 regions of the presumed promoters, and the regions with complementary symmetry are marked. The possible cleaving site for signal peptidase is between nucleotides 498-499.

Figure 1B:

Nucleotide sequence of the fragment encoding the *S. suis* type 2 *ef** gene of strain 1890 and the deduced amino acid sequence of the EF* protein of class I. The putative ribosome binding site, the -35 and -10 regions of the putative promoter sequences, the repetitive regions R1 - R11, and the putative termination signals are indicated. The region between the nucleotides 2859 and 5228 is absent in the gene encoding the 110 kDa EF protein. The region between the nucleotides 3423 and 4456 is absent in the genes encoding the class IV and class V EF* proteins.

Figure 2:

Nucleotide sequence of the 4.6 kb *Eco*RI-*Hin*dIII fragment with the *mrp* gene of *S. suis* type 2 and the MRP amino acid sequence derived therefrom. The probable ribosome binding site, the -35 and -10 regions of the presumed promoter sequences, the region of complementary symmetry beyond the *mrp* gene, the putative cleaving site for signal peptidase, the proline-rich region, the repeating amino acid sequences and the envelope anchor region are indicated.

Figure 3:

Restriction maps of *ef* containing fragments, subcloned into the plasmid vector pKUN19 (*24*). The open reading frames are boxed. Restriction sites: B:*Bam*HI; Bg:*Bgl*II; E:*Eco*RI; K:*Kpn*I; N;*Nar*I; P;*Pst*I; S;*Sna*BI; Sa:*Sal*I; Sp:*Spe*I.

Figure 4:

Schematic representation of the gene encoding the 110 kDa EF protein and the flanking regions. EF is encoded by the open reading frame 1 (ORF1). The 3' end of ORF1 is overlapping with the 5' end of ORF2. ORF2 and ORF3 are separated by a TAA stop codon. Restriction sites of interest are indicated.

Figure 5:

Schematic representation of the *Pst*I-*Sna*BI fragment of the *ef** genes of 5 different classes of the *ef* gene. The arrows indicate the repeated amino acid units. The lines indicate regions present in the different strains. The gaps indicate the regions lacking in the different strains.

Figure 6:

Nucleotide sequences near the ends of the fragments lacking in the *ef** genes of class IV and V (A) and in the *ef* gene (B). The uppermost and middle sequences represent regions flanking the left and right ends of the lacking fragments. The bottom sequences show the junctions as found in the class IV and V *ef** genes (A) and in the *ef* gene (B). Directly repeated sequences are shown in boxes. The bold nucleotides indicate the first bases of the translational triplets. The numbers refer to the nucleotide positions in the *ef** gene of class I (Fig. 1B).

Figure 7:

A. Restriction maps of the DNA inserts of putative MRP-positive recombinant bacteriophages. The thick line indicates the DNA region which is present in all of these clones. Restriction sites: E:*Eco*RI; H:*Hin*dIII; X:*Xba*I; K:*Kpn*I; S:*Sac*I. B. Parts of the DNA inserts subcloned in the plasmid vector pKUN19 (*24*).

Figure 8:

Western blot analysis of proteins, encoded by recombinant plasmids and recombinant bacteriophages, which have been s lect d with monoclonal antibodies against MRP. Lane 1: negative control; proteins extracted from the cell wall of a MRP-negative strain of *S. suis*. Lane 2: crude MRP preparation which contains proteins extracted from the cell wall of strain D282. Lane 3: pMR7-1. Lane 4: pMR7-2. Lane 5: pMR9-1. Lane 6: pMR9-2. Lane 7: pMR10-1. Lane 8: pMR10-2. Lane 9: lambda GEM11 with control insert. Lane 10: lambda clone 7. Lane 11: lambda clone 9. Lane 12: lambda clone 10. Lane 13: lambda clone 11.

Figure 9:

Western blot of the protoplast supernatant (PPS), culture supernatant (Cult. Sup.), and membrane vesicle (Membr.) fractions probed with anti-MRP/EF rabbit K191 serum (diluted 1:500). The lane designations are numbered strain designations.

Figure 10:

Western blot of cell culture supernatants of selected *S. suis* type 2 strains probed with rabbit anti-MRP/EF serum (K191), anti-MRP serum, and anti-EF serum (1:500 diluted). The PAbs revealed three *S. suis* type 2 phenotypes: $MRP^{+}EF^{+}$, $MRP^{+}EF^{-}$ and MRP^{-} EF^{-}. The lane designations are strain designations. Reference strain 1 (D-282) and strains 3 to 9 ($MRP^{+}EF^{+}$) were isolated from pigs with *S. suis* meningitis. Reference strain 2 (T-15) and strains 10, 12, 16, and 17 were isolated from the tonsils of healthy pigs. Strains 22, 23, 24, 25, 26, 28, and 29 were isolated from human patients.

Figure 11:

Hydropathy profile (25) of MRP. Sequences above and below the line represent hydrophobic and hydrophilic regions respectively.

Figure 12:

Homology between the amino acid sequences at the C terminus of MRP and several cell-envelope associated proteins of gram-positive bacteria. The amino acid sequence of *S. suis* MRP was compared with M6 protein of *Streptococcus pyogenes* (20), protein A of *Staphylococcus aureus* (16), protein G of group G streptococci (10), AP4 of *S. pyogenes* (13), LP of *Lactococcus lactis* (46), WAP4 of *S. mutans* (11), T6 of *S. pyogenes* (38), and Fn-BP of *S. aureus* (39).

Figure 13:

Comparison of the amino acid sequence of the repeat units in MRP. Homologous regions are enclosed in boxes.

Figure 14:

Fragments of the *mrp* and *ef* genes that were used as a probe. On top of each figure is the localisation of restriction sit s that were used to create the probes. The fragments which were used as probes are indicated with solid bars. Left of the solid bar is the abbreviation of the probe. The arrow indicates the open reading frame (ORF) of each gene. Fig. 14a: Probes of the *mrp* gene. The *Sac*I and *Hin*dIII sites are not authentic but are generated by subcloning fragments of the *mrp* gene. Fig. 14b: Probes of the *ef* gene. Fig. 14c: Probe of the *ef** gene. The open bar indicates the insert sequence of *ef** that is not part of the *ef* gene.

Figure 15:

Specificity of PCR. 10 ng of chromosomal DNA of *S. suis* type 2 strains was used in the PCR with the primers p-15, p-16, p-34, and p-35. Lanes 1 to 4 contained amplified DNA of MRP$^+$EF$^+$ strains (D282, 3, 10, and 22), lanes 5, 6, 7, and 9 of MRP$^+$EF* strains (17, 24, 26, 28), lanes 10 to 14 of MRP$^-$EF$^-$ strains (T15, 12, 16, 18, and 25), and lane 15 contained the negative control; all ingredients except DNA. Lanes 8 and 16 contained 300 ng size marker Lambda DNA digested with *Hin*dIII and *Eco*RI.

Figure 16:

Dot spot hybridization of 13 *S. suis* type 2 strains with the *mrp* and *ef* probes. In each experiment, row A contains 1 µg/spot DNA of four MRP$^+$EF$^+$ strains; D282, 3, 10 and 22, and one positive control. Row B contains four MRP$^+$EF* strains: strain 17, 24, 26 and 28; and row C five MRP$^-$EF$^-$ strains; T15, 12, 16, 18 and 25.

EXAMPLE 1

Cloning and nucleotide sequence analysis of the gene encoding the 110 kDa extracellular protein of pathogenic *Streptococcus suis* type 2 strains

MATERIALS AND METHODS

Bacterial strains and growth conditions.

E. coli strains JM101 (*29*) and LE392 (*33*) were used as hosts for recombinant plasmids and bacteriophages. The pathogenic MRP$^+$EF$^+$ strain D282 of *S. suis* type 2 (*43*) was used for the isolation of chromosomal DNA. *E. coli* strains were grown in Luria broth (*30*). Ampicillin was added as needed to a final concentration of 50 µg/ml. *S. suis* strains were grown in Todd-Hewitt broth (Oxoid, Ltd., London, England).

Construction and immunological screening of the DNA library. A DNA library of *S. suis* type 2 strain D282 was constructed in LambdaGEM-11 as

recommend d by th manufacturer of the cloning vector (Promega, Madison, USA). Recombinant bacteriophages were plated on *E. coli* strain LE392 and incubated for 16 h at 37°C.

Nitrocellulose filters (Schleicher and Schuell, Inc., Dassel, Germany) were placed on the plaques, and the plates were further incubated for 2 h at 37°C. Recombinants that produced EF were visualized with monoclonal antibodies (Mabs) directed against EF (Example 4). Bound antibodies were detected with anti-mouse serum conjugated with alkaline phosphatas (Zymed Laboratories, Inc., San Francisco, USA) as described by Maniatis et al. (*28*). Selected EF positive clones were purified by several rounds of single plaque isolation and immunological screening.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Proteins were separated by SDS gel electrophoresis in which 4% stacking and 6% separating gels were used (*26*). The separated proteins were transferred to nitrocellulose in a Semi-Dry transfer cell (Bio-Rad Laboratories, Richmond, USA). Specific proteins were visualized by use of polyclonal antibodies (Pabs, Example 4) or Mabs directed against EF and anti-rabbit or anti-mouse sera conjugated with alkaline phosphatase (Zymed Laboratories).

DNA manipulations and nucleotide sequence analysis. Selected restriction fragments were (sub)-cloned in the plasmid vector pKUN19 (*24*) by standard molecular biological techniques (*28*). Progressive unidirectional deletions were made with the Erase-a-Base system from Promega (Madison, USA). DNA sequences were determined by the dideoxy chain termination method (*37*). DNA and protein sequences were analysed by the software packages PCGENE (Intelli-genetics Corp., Mountain View, CA) and Wisconsin GCG (University of Wisconsin).

RESULTS

Cloning of the *ef* gene. A DNA library was constructed by isolating chromosomal DNA from strain D282 of *S. suis* type 2. This DNA was partially digested with the restriction enzyme *Sau*3A and cloned into the bacteriophage LambdaGEM11 replacement vector. The library contained approximately 5 x 10^5 recombinants per µg of DNA. Two thousand plaques of recombinant phages were tested for the presence of antigenic determinants of EF by use of a Mab directed against EF. Two plaques were positive.

The expression of EF by the two selected recombinant bacteriophages was studied by Western blotting to analyse the proteins eluted from plaques. Both recombinants encoded a protein that comigrated with EF secreted by *S. suis* and that was recognized by Mabs directed against EF. Thus both

recombinant bacteriophages contained the complete genetic information for EF. The genetic information for EF on the recombinant bacteriophages was localized using restriction enzyme analysis. The two clones shared a DNA region of about 13 kb. Parts of the common DNA region were subcloned into plasmid pKUN19 (Fig. 3) and the proteins expressed by the recombinant plasmids were analyzed by Western blotting. The plasmid containing the 6.8 kb *Kpn*I-*Sal*I fragment (pEF2-19, Fig. 3) encoded a protein with a molecular weight identical to EF, that was recognized by Mabs directed against EF. Plasmids containing the 5.8 kb *Eco*RV-*Sal*I or the 5.3 kb *Bgl*II-*Sal*I fragment, however, did not express EF. These data indicate that the *Eco*RV and the *Bgl*II sites are within regions required for EF expression.

<u>Nucleotide sequence of the *ef* gene</u>. The nucleotide sequence of the fragment comprising the EF encoding region was determined. The sequence (Fig. 1A) showed the presence of 3 major open reading frames (ORFs). ORF1 (from nucleotide 361 to 2890), ORF2 (from nucleotide 2856 to 3459) and ORF3 (from nucleotide 3462 to 4053) encoded polypeptides of 843 amino acids, of 201 amino acids and of 197 amino acids respectively. ORF1 contained a putative ATG start codon that is preceded by a sequence that is similar to ribosome binding sites of several types of gram-positive bacteria (*17*). In contrast, neither a start codon, nor a ribosome binding site upstream of the ORFs 2 and 3 could be found. The 3' end of ORF1 and the 5' end of ORF2 are overlapping, albeit in different frames. The ORFs 2 and 3 are separated by a single TAA stop codon. Upstream of ORF1 two putative promoter sequences were found that resembled the -35 and -10 consensus sequences of promoters commonly found in gram-positive bacteria (Fig. 1A). Downstream of ORF3, two regions of extented dyad symmetry were present. Because both regions contained a stretch of thymidine residues at the end of the potential stem-loop structures, these potential transcription terminators are likely to be rho-independent (*34, 40*). Because the sequence data did not reveal obvious transcription and translation signals upstream of, or within ORF2 and ORF3, it is doubtful that these ORFs express proteins. Another possibility is that the entire sequenced region contains one large open reading frame. This situation would occur if only two sequence errors were present: a +1 base pair frame shift in the region 2856 to 2892 and an error in the stop codon at position 3459. This possibility was excluded by sequencing the *ef* gene from three additional, independently selected clones. Fragments of the initial clones were used as hybridization probes in order to isolate

these clones from the chromosome. The nucleotide sequences f these fragments were identical to thos presented in Fig. 1A.

Amino acid sequence of EF. Because only ORF1 was preceded by appropriate expression/initiation signals, this ORF probably encodes EF. This was confirmed by subcloning two fragments into plasmid pKUN19: a *Spe*I-*Sna*BI fragment, that contained the entire ORFs 1 and 2 and a *Spe*I-*Nar*I fragment, that contained ORF1 and the 5' end of ORF2 (Fig. 3). The proteins expressed by the recombinant plasmids were analysed by Western blotting. In *E. coli* both recombinant plasmids encoded a protein that was recognized by a Mab directed against EF and that had a molecular weight identical to that of EF secreted by *S. suis*. Therefore, ORF1 encodes EF. The molecular weight of the ORF1 product calculated from the sequenc (90,000) differed, however, from that of EF estimated from SDS polyacrylamide gels (110,000).

EF is exclusively found in the supernatant of *S. suis* cultures, and thus the protein is expected to be preceded by a signal peptide. Indeed, th first 46 amino acids of the deduced amino acid sequence of EF are characteristic of a typical signal peptide. An N-terminal part that contained six positively charged amino acids was followed by a hydro-phobic cor of 21 amino acids and a putative signal peptidase cleavage site (*45*). The hydropathy pattern (*25*) of the deduced amino acid sequence showed that, apart from the signal peptide, the EF protein was very hydrophilic and did not contain extended hydrophobic regions (cf. MRP, Example 3). No significant similarities were found between the deduced amino acid sequence of EF and the protein sequences in the EMBL Data Library.

Although appropriate translation initiation signals upstream of ORF2 and ORF3 could not be found, the deduced amino acid sequences of ORF2 and ORF3 showed some properties which raised doubt to the idea that those frames are not expressed. The N-terminus of the putative ORF2 protein showed two highly repetative units of 57 amino acids (identity 82%). The C-terminus of the putative ORF3 protein is functionally similar to C-terminal regions of several cell-envelope located proteins of gram-positive bacteria (*10, 12, 13, 16, 41*). A hydrophobic region was preceded by the conserved sequence Leu-Pro-X-Thr-Gly-Glu and followed by a highly hydrophilic region. This similarity suggests that the putative ORF3 protein is associated with the cell-envelope.

EXAMPLE 2

Cloning and nucleotide sequence analysis of genes encoding extracellular proteins of non-pathogenic *Streptococcus suis* type 2 strains

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain JM101 (*29*) was used as host for recombinant plasmids. Seventeen MRP$^+$EF* strains of *S. suis* type 2 were isolated from human patients, five strains from tonsils of slaughthered pigs, seven strains from organs of diseased pigs and from two strain the origin was unknown (Example 4). The *E. coli* strain was grown in Luria broth (*30*). Ampicillin was added as needed to a final concentration of 50 μg/ml. *Streptococcus suis* strains were grown in Todd-Hewitt broth (Oxoid, Ltd., London, England).

Genomic DNA and oligonucleotides. Genomic DNA was isolated by lysis in proteinase K/SDS solution, extraction with phenol/chloroform and precipitation with ethanol (*28*). The sequences of the oligonucleotides used in the polymerase chain reaction (PCR) were: 5'-ATGTAATTGAATTCTCTTTTTAAGT-3' and 5'-AAACGTCCGCAGACTTCTAGATTAAAAGC-3'. These oligonucleotides correspond to the positions 35 to 59 and 4308 to 4279 in the *S. suis* type 2 *ef* gene. The underlined sequences indicate the recognition sites for the restriction enzymes *Eco*RI and *Xba*I.

DNA manipulations and nucleotide sequence analyses were carried out as described in Example 1.

SDS - PAGE and Western blot analysis were carried out as described in Example 1.

Southern hybridization. DNA was transferred to Gene-Screen Plus membranes (New England Nuclear Corp., Dreieich, Germany) as described by Maniatis et al. (*28*). DNA probes were labeled with (^{32}P)dCTP (3000Ci/mMol, Amersham Corp., Arlington Heights, USA) by the use of a random primed labeling kit (Boehringer GmbH, Mannheim, Germany). The blots were hybridized with DNA probes as recommended by the supplier of the Gene-Screen Plus membranes. After hybridization the membranes were washed twice with a solution of 2 x SSC (1 x SSC is 0.15M NaCl plus 0.015 M trisodium citrate, pH 7.0) for 5 min at room temperature and twice with a solution of 0.1 x SSC plus 0.5% SDS for 30 min at 65°C.

Amplification of genomic DNA fragments by Polymerase Chain Reaction (PCR). PCR was used to amplify *ef** sequences. Genomic DNA from different MRP$^+$EF* strains of *S. suis* type 2 was used as a template. Amplified DNA fragments were isolated by agarose gelelectrophoresis and extraction from the gel with Gene Clean (Bio101, La Jolla, USA). The purified fragments

w re digested with *Eco*RI and *Xba*I and cloned into th plasmid pKUN19 (*24*). To exclude mistakes in the DNA sequences as a result of the PCR, six independently choosen clones were mixed prior to the nucleotid sequence analyses.

RESULTS

<u>Western blot of EF* proteins</u>. Culture supernatants of strains of *S. suis* type 2 belonging to the MRP+EF* phenotype contained proteins that were recognized by Mabs directed against EF (Examples 4, 6). The molecular weights (MW) of these proteins varied and were higher than that of EF. The proteins secreted by thirty-one strains of the MRP+EF* phenotype were compared with those secreted by a strain of the MRP+EF+ phenotype. EF* proteins of five different molecular weight classes were found. Three strains synthesized an EF* protein of approximately 195 kDa (class I); eighteen an EF* of approximately 180 kDa (class II); one an EF* of approximately 175 kDa (class III); five an EF* of approximately 160 kDa (class IV) and four an EF* of approximately 155 kDa (class V).

<u>Southern hybridization of *ef** genes</u>. The relationship between the genes encoding the 110 kD EF and the EF* proteins was studied. Chromosomal DNA of different MRP+EF* strains (two representatives of each class were taken) and of the MRP+EF+ strain D282 (*43*) was digested with the restriction enzyme *Pst*I. The various DNAs were hybridized with a ^{32}P labeled *Eco*RV-*Sna*BI fragment containing the entire *ef* gene (Fig. 4, see Example 1). The results showed that the DNA digests of the MRP+EF* as well as the MRP+EF+ strains contained two *Pst*I fragments that strongly hybridized with the probe. These data indicated that the genes encoding the 110 kDa EF and the EF* proteins are strongly related. The length of the largest hybridizing fragment was the same in all strains. In contrast, the length of the smallest hybridizing fragment differed between the strains. Moreover, the variation in length of the smallest hybridizing fragment correlated well with the variation in the molecular weight of the EF* proteins secreted by the different strains. Since the smallest hybridizing fragment is located at the 3' end of the *ef* gene (Fig. 4, Example 1), these data suggest that the *ef* and *ef** genes differed mainly at their 3' ends.

<u>Cloning of *ef** genes</u>. The genes encoding the different EF* proteins were obtained using PCR to amplify the *ef** containing DNA fragments. Genomic DNA of 5 different MRP+EF* strains of *S. suis* type 2 (one representative of each class) was used as a template. The amplified fragments were digested with restriction enzymes *Eco*RI and *Xba*I and cloned into *E. coli*.

Ef* gene of class I. The nucleotide sequence of a 6.8 kb *Eco*RI-*Xba*I fragment containing the entire *ef** gene of class I and the regions flanking it was determined. Analysis of the sequence revealed two open-reading frames (ORFs, Fig. 1B). The first ORF (from nucleotide 361 to 5827) and the second ORF (from nucleotide 5830 to 6421) encoded polypeptides of 1822 amino acids and 197 amino acids respectively. Based on its size the first ORF is expected to encode the EF* protein (195 kDa). The ORFs were separated by a single TAA stop codon. The first ORF contained a putative ATG start codon that was preceded by a sequence similar to bacterial ribosome-binding sites (*17*). In contrast, the second ORF was not preceded by an appropriate start codon, nor by a putative ribosome-binding site.

The first 46 amino acids of the deduced amino acid sequence of the EF* protein had the characteristics of a typical signal peptide (*45*). The C terminus of the mature part of the protein contained a number of imperfect repeats of 76 amino acids. In the EF* protein of class I ten and a half repeats were present (denoted as R1 to R11, Fig. 1B). The first four repeats were contiguous as were the last six and a half repeats. The fourth and the fifth repeated unit, however, were separated by 113 amino acids and the fifth and the six unit by 22 amino acids (Fig. 5). The amino acid sequences of the last five and a half unit were highly conserved, whereas the sequences of the first five units were more variable. One particular amino acid sequence, Asn-Pro-Asn-Leu, was conserved in all repeated units. No significant homology was found between the EF* sequence of class I and any protein sequence in the EMBL Data Library.

Ef* genes of class II, III, IV and V. Because the genes encoding the various EF* proteins differed mainly at their 3' ends, the nucleotide sequences of the small *Pst*I fragments from the genes of class II, III, IV and V were determined. Comparison of the nucleotide sequences showed that the various *ef** genes were highly homologous in this region. The *ef** genes differed, however, in the number and the arrangement of repeated units (Fig. 5). Unlike the *ef** gene of class I, the *ef** genes of class II and IV lacked the R9 and R10 regions; that of class III lacked the R6, R7 and R9 regions and that of class IV lacked the R7, R8 and R9 regions. In addition, the *ef** genes of class IV and V lacked a fragment of 1,032 bp, which contained R4, R5 and parts of R3 and R6. The translational reading frame of the region located at the 3' end of the missing fragment remained the same. The nucleotide sequences at the regions of the left

and right ends of this 1,032 bp fragment showed direct repeats of 9 bp (Fig. 6A).

Homology between *ef** and *ef* genes. Because EF* proteins were recognized by Mabs directed against the 110 kDa EF protein and because the *ef** genes strongly hybridized with an *ef*-probe, the *ef* (Example 1) and *ef** genes are assumed to be partly identical. Comparison of the nucleotide sequences of the *ef* and the *ef** gene of class I showed that the 2,499 nucleotides located at the 5' end of the *ef* and *ef** encoding regions were identical. Unlike the gene encoding the EF* protein of class I, the gene encoding the 110 kDa EF protein lacked a 2,368 bp fragment. As a result of this deletion the reading frame was altered and the region located at the 3'-end of the 2,368 bp fragment was translated in different frames in *ef* and *ef** genes. Consequently, the 110 kDa EF protein will not contain the repeated amino acid units. Analysis of the nucleotide sequences at the regions of the left and right ends of the 2,368 bp fragment showed direct repeats of 10 bp (containing one mismatch) (Fig. 6B). Thus, the gene encoding the 110 kDa EF protein could have been the result of a specific deletion of 2,368 bp within an *ef** gene. This would implicate that a *S. suis* strain that is non-pathogenic can change into a strain that is pathogenic.

EXAMPLE 3

Cloning and nucleotide sequence of the gene encoding the 136 kDa surface protein (MRP) of *Streptococcus suis* type 2

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain JM101 (*sup*E, *thi*, (*lac-pro*AB$^-$)[*F'tra*D36, *lac*I^{q}ZΔM15], *29*) was used as a host for recombinant plasmid DNA. *E. coli* strain LE392 [F$^-$*hsd*R574(rk$^-$mk$^+$), *sup*E44, *sup*F58, *lac*Y1, or Δ(*lac*IZY)6, *gal*K2, *gal*T22, *mel*B1, *trp*R55] (*33*) was used as a host for recombinant bacteriophages. The pathogenic MRP$^+$EF$^+$ strain D282 of *S.suis* type 2 (*43*) was used for isolating chromosomal DNA. *E. coli* strains were grown on LB broth (*30*). Solid LB medium contained 1.5% agar. Ampicillin was added as needed to a final concentration of 50 µg/ml. *Streptococcus suis* strains were grown in Todd-Hewitt broth (Oxoid Ltd.)

Southern hybridization was carried out as described in Example 2.

Construction and immunological screening of the DNA library were carried out as described in Example 1 substituting MRP for EF.

SDS - PAGE and Western blot analysis were carried out as described in Example 1 substituting MRP for EF.

Nucleotide sequence analysis was carried out as described in Example 1.

RESULTS

Construction and screening of the library. Chromosomal DNA isolated from strain D282 of *S. suis* type 2 was partially digested with the restriction enzyme *Sau*3A. A DNA library was then constructed in the bacteriophage LambdaGEM11 replacement vector. Approximately 5×10^5 recombinants/μg DNA were obtained. A MAb directed against MRP was used to screen 1,400 recombinant plaques for the presence of antigenic determinants of MRP. Five recombinant plaques reacted positive.

Characterization of the immunoreactive recombinants. The expression of MRP by the five selected recombinant bacteriophages was studied by Western blotting to analyse the proteins eluted from the plaques. All five recombinants encoded proteins that were recognized by MAbs directed against MRP. These proteins, however, had lower molecular weights (MW) than the MRP. Two clones encoded a protein of approximately 70 kDa (clones 10 and 11); two clones encoded a protein of approximately 80 kDa (clones 9 and 12), and one clone encoded a protein of approximately 90 kDa (clone 7). Therefore, it was concluded that the five recombinants did not contain the complete genetic information for MRP. Restriction enzyme analysis was used to compare the DNA inserts of the five recombinants. All clones shared a DNA region of about 17 kb (Fig. 7A). The DNA inserts differed, however, at the 3' and 5' ends. The variation in length at the 3' ends of the inserts correlated well with the variation in MW of the truncated MRP proteins (cf. Fig. 7A). This correlation indicates that MRP encoding sequences were located at the 3' end of the DNA inserts. This was confirmed by subcloning fragments derived from the 3' end of the DNA inserts of clones 7, 9, and 10 (Fig. 7B) into plasmid vector pKUN19 (*24*). These constructs encoded truncated MRP proteins that were indistinguishable from the truncated MRP proteins encoded by the recombinant phages (Fig. 8). Deletion of the 0.7 kb *Eco*RI-*Kpn*I fragment from these contructs stopped the expression of the truncated MRP proteins. This suggests that the expression of *mrp* is initiated from the 0.7 kb *Eco*RI-*Kpn*I fragment.

Cloning of the complete *mrp* gene. The complete gene for MRP was obtained by hybridization of the ^{32}P labeled *Kpn*I-*Sac*I fragment of pMR7-2 (Fig. 7B) with *Eco*RI or *Kpn*I digested chromosomal DNA of strain D282 of *S. suis* type 2. An *Eco*RI fragment of 7 kb and a *Kpn*I fragment of 7 kb hybridized

with th probe. Becaus of its size, the *Eco*RI fragment was expected to contain the compl te *mrp* gene and because the expression of *mrp* is initiated from the 0.7 kb *Eco*RI-*Kpn*I fragment, the *Kpn*I fragment was expected to contain only the 3' end of the gene. Fragments ranging from 6 to 8 kb from *Eco*RI and *Kpn*I digested chromosomal DNA were isolated, and ligated into the *Eco*RI or *Kpn*I site of pKUN19, whereafter the ligation mixtures were transformed into *E. coli* JM101. Thirteen out of 50 selected recombinant clones obtained with the *Kpn*I fragments hybridized with a MRP probe. All of these recombinant clones contained a plasmid (pMR-C) with a 7 kb *Kpn*I insert. In contrast, of 2,500 selected recombinant clones obtained with *Eco*RI fragments, none hybridized with the probe. Since the 7 kb *Eco*RI fragment is expected to contain the complete *mrp* gene, this finding indicates that expression of MRP is toxic in *E. coli*. Nevertheless, a plasmid (pMR11) with the entire *mrp* gene could be constructed by combining the 5' end of the *mrp* gene (isolated from pMR7-2) and the 3' end of the gene (isolated from pMR-C) by forced cloning. The copy number of this plasmid appeared to be strongly reduced, about 20 times, compared to the copy number of pKUN19. The low copy number presumably reduced the toxic effects of high-level expression of MRP in *E. coli* to tolerable levels. The proteins produced by *E. coli* cells containing pMR11, were analysed by Western blotting. As expected, these cells produced a 136 kDa protein that comigrated with MRP and that was recognized by PAbs directed against MRP.

<u>Nucleotide sequence of the *mrp* gene</u>. The nucleotide sequence of a 4.6 kb *Eco*RI-*Hind*III fragment, containing the entire *mrp* gene and the regions flanking it was determined. Analysis of the sequence, Fig. 2, revealed an open reading frame of 3,768 nucleotides coding for a polypeptide of 1,256 amino acids (with a calculated MW of 135,794). The putative ATG start codon is preceded by a sequence that is similar to ribosome-binding sites in several types of gram-positive bacteria (*17*). The nucleotide sequence upstream of *mrp* resembles the -35 and -10 consensus sequences of promoters commonly found in gram-positive bacteria. Downstream of the *mrp* gene, a region showing extended dyad symmetry can be detected. The potential hairpin structure in the corresponding mRNA has a 12 bp stem separated by a 6 bp loop (ΔG = -15.9 kcal/mol, calculated according to the rules of Tinoco et al., *40*). Since the region of dyad symmetry is not followed by a thymidine-rich region, this potential transcription terminator signal appears to be rho-dependent (*34*).

Amino acid sequence of MRP. MRP is a cell-envelop associated protein and must be translocated across the cytoplasmic membrane. The mature protein must therefore contain a signal peptide. Indeed, the first 47 amino acids of the MRP have the characteristics of a typical signal peptide. An N-terminal part that contains seven positively charged residues is followed by a hydrophobic core of 21 amino acids and a putative signal peptidase cleavage site (*45*, vertical arrow in *Fig. 2*). Cleavage of the signal peptide would result in a mature protein with an MW of 131,094, which is close to the MW (136 kDa) of MRP, estimated from SDS-polyacrylamide gels (Example 4). A second hydrophobic region of 20 amino acids was identified at the C terminus of the protein (Fig. 11). If this region is analogous to other envelope associated proteins of gram-positive bacteria (*10, 11, 12, 13, 16, 20, 38, 39, 46*), it is probably a cell membrane anchor. A short highly charged region and a region with the Leu-Pro-X-Thr-Gly-Glu amino acid sequence, two regions that flank the presumed cellmembrane anchor, are also highly conserved among surface proteins of gram-positive bacteria (Fig. 12). The amino acid sequence Leu-Pro-X-Thr-Gly-Glu is putatively involved in cell-wall binding.

Several other regions were identified in the MRP sequence. The mature form of MRP starts with a unique N-terminal sequence of 824 amino acids. This region is followed by a stretch of amino acids that is rich in proline residues: of 86 amino acids, 26 are proline residues. This region is followed by three repeated units of 54 amino acids (Fig. 13). The first unit is separated from the second by 77 amino acids, but the second and third unit are contiguous. The sequences of the first and the second unit are highly conserved, whereas the third varies. The third repeated unit is followed by the envelope anchor sequence. There was little homology between the MRP sequence and the protein sequences of the EMBL Data Library. One subsequence of MRP, amino acid residues 619 - 985, however, shared some similarity (17.2% identity in a 377 amino acids sequence) with a sequence of the fibronectin-binding protein of *Staphylococcus aureus* (*39*).

EXAMPLE 4

Identification of two proteins associated with virulence of *Streptococcus suis* type 2

MATERIAL AND METHODS

Streptococcal isolates. 180 strains of *S. suis* type 2 were obtained from three different sources. A total of 111 of these strains were obtained

from four Animal H alth S rvices in the Netherlands. Thes strains were isolated from organs of diseased pigs in the course of routine diagnostic procedures. Another 42 strains were isolated from tonsils of h althy pigs when they were slaughtered. 27 strains were isolated from human patients with *S. suis* type 2 infections. Tonsillar and human strains were kindly provided by J.P. Arends, Streeklaboratorium voor de Volksgezondheid voor Groningen en Drente, Groningen, the Netherlands. All strains were typed as *S. suis* type 2 by using biochemical and serological methods, as described previously (*44*). Strain 1 (= D282) had been determined previously to be virulent for newborn germfree pigs and produced MRP, whereas strain 2 (= T-15) was nonvirulent and did not produce MRP (*43*). Therefore, strains 1 (MRP^+) and 2 (MRP^-) were used as reference strains.

Culture conditions. A 1-day-old colony of each bacterial strain was grown on Columbia blood agar base (code CM 331; Oxoid, Ltd.) containing 6% horse blood and was incubated overnight at 37°C in Todd-Hewitt broth (code CM 189; Oxoid). Early stationary growth phase cultures were obtained from the overnight cultures, diluted 10 times in Todd-Hewitt broth, and incubated for 4 h at 37°C.

Cell fractionation. Two cell fractions (protoplast supernatant and culture supernatant) were prepared from each of the 180 strains. Two more cell fractions (protoplasts and membrane vesicles) were prepared from 23 strains selected randomly from the 180 strains. The 23 strains were isolated from both diseased and healthy pigs, as well as from human patients. The four cell fractions were isolated from early stationary growth phase cultures in Todd-Hewitt broth. Protoplasts were isolated as described by Van der Vossen et al. (*47*). After centrifugation in an Eppendorf centrifuge, the protoplasts and the remaining supernatants (protoplast supernatant) were collected. Membrane vesicles were isolated as decribed by Driessen et al. (*9*). The broth cultures were centrifuged at 4,000 x *g* for 15 min, and the culture supernatants were collected.

Preparation of antigens and antisera. After a stationary growth phase culture of strain D-282 was centrifuged, the supernatant was harvested, concentrated by filtration (type PM30 filters; Amicon Corp., Danvers, Mass.) to a concentration of 3 mg/ml, and dialysed once against Tris-buffered saline (50 mM, pH 7.5). This product was used as an antigen for raising polyclonal antibodies (PAb) in rabbits and monoclonal antibodies (MAb) in mice. Rabbits were immunized by intramuscular and subcutaneous inoculation of 2 mg portions of protein emulsified in qual volumes of Freund imcomplet adjuvant. Inoculations were repeated the following day

without the adjuvant. After 5 weeks the rabbits were given intravenous booster inoculations of the same antigen dose, but without the adjuvant. After 6 weeks, the rabbits were exsanguinated. The serum of one rabbit (rabbit K191) was used as a probe in the Western blot analysis.

MAbs against the protein EF were raised in BALB/c mice. The mice were immunized intraperitoneally with 0.5 ml portions of antigen containing 25 µg of protein emulsified in equal volumes of Freund imcomplete adjuvant; 3 weeks later this procedure was repeated. After 5 weeks, the mice were given intravenous booster inoculations of the same antigen dose, but without the adjuvant. Hybridoma cell lines were prepared as described by Van Zijderveld et al. (*51*). After 10 to 14 days, hybridomas were tested for antibodies against EF by using an enzyme-linked immunosorbent assay. Hybridoma culture supernatants (diluted 1:2) were then tested for anti-EF MAb on Western blots of culture supernatants from strain D-282. Binding of MAb to the 110 kDa protein on the nitrocellulose filters was visualized with anti-mouse immunoglobulins conjugated with alkaline phosphatase. The positive cells were cloned twice by limiting dilution in microtiter plates. The resulting monoclonal cell lines were used to produce ascites fluid in pristane-primed male BALB/c mice, as described previously (*51*).

Indirect enzyme-linked immunosorbent assay for screening hybridoma culture supernatants. Polystyrene microtiter plates (Greiner, Nürtingen, Germany) were coated for 16 h at 37°C with a solution containing the concentrated, dialysed culture supernatant from strain D-282 (see above) diluted in phosphate-buffered saline (pH 7.2; 0.075 mg of protein per ml), and these preparations were incubated for 16 h at 37°C. Twofold dilutions of hybridoma culture supernatants were applied and tested as described previously (*51*). Bound antibodies were incubated with anti-mouse immunoglobulins (diluted 1:500) that were conjugated with horseradish peroxidase (HRPO, Nordic, Tilburg, The Netherlands).

Electrophoresis and Western blotting. The various cell fractions were analysed by SDS-PAGE as described by Laemmli (*26*) on 6 or 12% polyacrylamide. After electrophoresis, the proteins were stained with silver (*32*). For Western blot analysis, the proteins were electroblotted onto nitrocellulose by using a Multiphor II Nova Blot system (Pharmacia LKB, Uppsala, Sweden). The blots were probed with a 1:500 dilution of rabbit K191 PAb or with a 1:300 dilution of mouse MAb. Bound PAb were visualized with anti-rabbit immunoglobulins conjugated with alkaline phosphatase. Bound MAb were visualized with a 1:1,000 dilution of anti-mouse

immunoglobulins conjugated with alkalin phosphatase (Zymed).

RESULTS

Protein profiles of four cell fractions of 23 selected strains. The protein profiles of the protoplast supernatants and membrane vesicle cell fractions from two *S. suis* isolates belonging to each group studied (diseased pigs, healthy pigs, and human patients), prepared from the 23 strains examined were almost identical. In contrast, the protein profiles of the culture and protoplast supernatants differed distinctly. The protein profiles of isolates obtained from diseased pigs contained two protein bands that were absent in the protein profiles of most isolates obtained from healthy pigs. One band represented a 136 kDa protein, which was identified as MRP (*43*). In the SDS-PAGE analysis, separating gels containing 6% polyacrylamide revealed the presence of MRP in both culture and protoplast supernatants (strains 1, 5, 24, and 26). The second band represented a 110 kDa protein; because this protein was detected only in culture supernatants, it was designated EF. Both MRP and EF were present in the culture supernatant of virulent reference strain 1 (= D-282), but were absent in all cell fractions of nonvirulent reference strain 2 (= T-15). The eight strains isolated from diseased pigs contained both MRP and EF. Six of the eight strains isolated from healthy pigs lacked these proteins. Six of the seven strains isolated from human patients contained MRP, but only three of the six also contained EF.

When rabbit K191 PAb directed against culture supernatants were used as probes in the immunoblotting analysis, MRP and EF were clearly detected in the cell fractions of *S. suis* type 2 strains. Protoplast supernatants, culture supernatants, and membrane vesicles of strains 1, 5, 24, and 26 contained the 136-kDa MRP (Fig. 9). Because MRP is a major component of protoplast supernatants, this protein must be localized in the cell envelope of the bacteria. The culture supernatants of strains 1 and 5 also contained the 110 kDa EF. Strains 24 and 26 contained MRP but not EF; strains 2 and 13 contained neither of the proteins.

On the basis of the presence of MRP and EF in culture supernatants, the following three phenotypes of *S. suis* type 2 strains were distinguished: $MRP^{+}EF^{+}$, $MRP^{+}EF^{-}$, and $MRP^{-}EF^{-}$ (Fig. 10). Proteins bands at various molecular masses higher than 150 kDa reacted with rabbit K191 serum and were visualized in Western blots of culture supernatants of strains 17, 24, 25, 26, and 28. As such proteins were also recognized by the anti-EF MAb, except in the culture supernatant of strain 25, the 110 kDa EF was probably related to these proteins. Western blots probed with th mouse

anti-EF MAb showed that all of the strains with the MRP$^+$EF$^-$ phenotype contained high r molecular w ight proteins in their culture supernatants. However, none of the strains with the MRP$^+$EF$^+$ phenotype contained such proteins. Probing with rabbit K191 serum revealed high molecular weight proteins in culture supernatants of 12 MRP$^-$EF$^-$ strains, including strain 25. Immunoblotting with anti-EF MAb showed that these proteins were not related to EF. When the four cell fractions were analysed by SDS-PAGE on 12% slab gels, no low molecular weight proteins associated with virulence were detected.

Protein profiles of culture and protoplast supernatants of 180 strains. All 180 *S. suis* type 2 strains were analysed for the occurrence of the three phenotypes in culture and protoplast supernatants by using 6% slab gels. Eighty percent of the strains isolated from the organs of diseased pigs had the MRP$^+$EF$^+$ phenotype (Table 1).

TABLE 1. Prevalence of MRP and EF phenotypes in 180 streptococcal strains isolated from diseased pigs, from healthy pigs when they were slaughtered, and from human patients.

S. suis type 2 phenotype	No. (%) of strains isolated from:		
	Organs of diseased pigs	Tonsils of healthy pigs	Human patients
MRP$^+$EF$^+$	86 (77)	1 (2)	4 (15)
MRP$^+$EF$^-$	13 (12)	5 (12)	20 (74)
MRP$^-$EF$^-$	12 (11)	36 (86)	3 (11)

In contrast, only 2% of the strains isolated from tonsils of healthy pigs had this phenotype; 86% of these strains were MRP$^-$EF$^-$. Only 15% of the strains isolated from human patients had the MRP$^+$EF$^+$ phenotype. Among the *S. suis* type 2 strains tested, far more human strains (74%) than porcine strains (12%) had the MRP$^+$EF$^-$ phenotype; 89% of the human strains were MRP$^+$. The MRP$^-$EF$^+$ phenotype was not detected.

EXAMPLE 5

Virulence of *Streptococcus suis* type 2 strains in new-born germ-free pigs.

MATERIALS AND METHODS

Pigs. Fifty-two germ-free pigs, cross-breeds of Great Yorkshire and Dutch Landrace, were obtained from four sows by caesarian sections. Sows in both experiments were full sisters. Pigs were allotted to 12 groups each consisting of 4 or 5 pigs. Each group was housed in a sterile stainless

steel incubator. Housing and feeding were as described before (*43*).

Inocula. Ten *S. suis* type 2 strains belonging to ither phenotype MRP+EF+, MRP+EF-, or MRP-EF- were obtained from three sources: from a pig with meningitis, from healthy pigs at slaughter, and from human patients (Table 2). The strains were biochemically and serologically typed as described earlier (*44*). Strains were stored as stock suspensions on glass beads in Nutrient Broth with 15% glycerol at -70°C. A one-day-old colony of each strain, grown on Columbia blood agar base (Code CM 331, Oxoid) containing 6% horse blood, was incubated overnight at 37°C in Todd-Hewitt broth (Code CM 189, Oxoid). Early stationary growth phase cultures were obtained by diluting the overnight cultures in Todd-Hewitt broth (1:10) and incubated them at 37°C. Incubation was stopped after approximately 4 h, when the optical density at 600 nm was 0.5. Cultures containing approximately 1 to 3 x 10^9 CFU/ml were then centrifuged at 4000 x g for 15 min. The supernatant was analysed for MRP and EF. Then the pellets were washed and suspended at an A_{600} = 1 in a solution of phosphate-buffered saline (PBS), 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 2.79mM KH_2PO_4, pH 7.2, and then used as *inoculum*. *Bordetella bronchiseptica* strain 92932, isolated from the nose of a pig with atrophic rhinitis, was used to predispose pigs to *S. suis* infection (*23*, *43*). The strain was kept on Dorset egg medium. The inoculum was prepared by culturing a 48 hour old colony from sheep blood agar in brain heart infusion broth. After 18 h of incubation at 37°C, this medium contained approximately 10^9 CFU/ml. The brain heart infusion broth was diluted (1:100) in PBS to prepare the inoculum.

Electrophoresis and Western blotting. The MRP/EF phenotypes of the *S. suis* strains used as inocula and of the isolates recovered at the end of the experiments were determined. SDS-PAGE as described by Laemmli (*26*) (6% polyacrylamide) and Western blotting were used to analyse cell culture supernatants of isolates recovered from nasopharynx of all pigs, and from inflamed tissues such as meninges or joints of affected pigs. After electrophoresis the proteins were stained with silver (*32*). For Western blot analysis, the proteins were electroblotted onto nitrocellulose by the Multiphor II Nova Blot system, according to the recommendations of the manufacturer (Pharmacia LKB). Nitrocellulose filters were incubated either with a 1:1 mixture of mouse anti-MRP monoclonal antibodies (MAb) (11.3 mg/ml) and anti-EF MAb (8.4 mg/ml) each in a 1:200 dilution, or with a 1:500 dilution of polyclonal anti-MRP/EF rabbit serum (K191) (8.2 mg/ml) (Examples 4, 6). Filters were incubated

with a 1:1000 dilution of anti-mouse immunoglobulins conjugated with alkaline phosphatase (AP) or a 1:3000 dilution of AP conjugated anti-rabbit immunoglobulin G (γ + κ) (Zymed). Bound antibodies were visualized by adding the substrate bromochloroindolyl phosphate (Sigma, St. Louis, Mo) - nitro blue tetrazolium (Merck, Darmstad, Germany) in phosphatase buffer (100mM NaCl, 5 mM $MgCl_2$, 100 mM diethanolamine; pH 9.5).

Experimental design. The study consisted of two experiments with an interval of five months. Five day old germ-free pigs were inoculated intranasally with a plastic disposable syringe filled with a suspension of *B. bronchiseptica* strain 92932 in brain heart infusion broth. The inocula contained 0.84 x 10^7 CFU in experiment I and 1.0 x 10^7 CFU in experiment II. Two days post inoculation (pi) the pigs were similarly inoculated inside the sterile incubator with one of the ten *S. suis* type 2 strains (Table 2).

The mean (± SD) inoculum size of these strains was 1.4 (+ 0.60) x 10^6 CFU. All inoculations consisted of a 0.5 ml bacterial suspension in each nostril during the inspiratory phase of breathing. In both experiments strain 3 (MRP+EF+) was used as positive control and strain 12 (MRP-EF-) was used as negative control (see Results section). Pigs were killed either when they became mortally ill or at the end of the experiment (3 to 4 weeks pi), and they were subsequently necropsied.

TABLE 2. Experimental design.

S. suis strain no.	*S. suis* phenotype	Source[1] of *S. suis* isolation	Dosage[2] of *S. suis* inoculation	No. of pigs inoculated
3	MRP+EF+	meninges pig	1.84	5
3	MRP+EF+	meninges pig	1.96	4
10	MRP+EF+	tonsil pig	1.52	5
22	MRP+EF+	human	2.93	4
17	MRP+EF-	tonsil pig	1.26	4
24	MRP+EF-	human	1.22	4
28	MRP+EF-	human	1.23	4
12	MRP-EF-	tonsil pig	1.05	5
12	MRP-EF-	tonsil pig	0.98	4
16	MRP-EF-	tonsil pig	0.70	4
18	MRP-EF-	tonsil pig	1.10	4
25	MRP-EF-	human	0.97	4

[1] Strain 3 was isolated during routine diagnostic procedures from a pig with meningitis. Strains 10, 12, 16, and 18 were isolated at slaughter from the tonsils of healthy pigs. Strains 22 (no. 830544), 24 (no. 740113), 25 (no. 821021) and 28 (no 760366) were isolated from human patients with *S. suis* type 2 meningitis. (Numbers between parentheses refer to those by J.P. Arends and H.C. Zanen (*2*)).

[2] x 10^6 CFU.

Disease monitoring. Pigs were monitored daily for clinical signs of disease, such as fever, dysfunction of the CNS and lameness. Blood samples from each pig were collected three times weekly by venipunctur of the cranial vena cava. White blood cells were counted with a conducting counter (Contraves A.G., Zürich, Switzerland) (*18*). The number of neutrophils was calculated after differential count of Giemsa-stained blood smears. Swabs specimens of nasopharynx and feces were collected daily and plated directly onto Columbia agar containing 6% horse blood. The presence of *S. suis* type 2 and of *B. bronchiseptica* was confirmed by slide agglutination test in which a suspension of the monocultures was mixed with the appropiate hyperimmune rabbit serum (DLO-Central Veterinary Institute, Lelystad, NL). After pigs were killed, they were examined for pathologic changes. Tissue specimens of the CNS, serosae, liver, spleen, and tonsils were bacteriologically and histologically examined as described before (*43*).

RESULTS

Electrophoresis and Western blotting. When immunoblots were used to analyse culture supernatants of the *S. suis* strains before inoculation, three phenotypes were distinguished. Strains 3, 10, and 22 belonged to the MRP+EF+ phenotype, strains 17, 24, and 28 were of the MRP+EF- phenotype, and strains 12, 16, 18, and 25 belonged to the MRP-EF- phenotype. The rabbit polyclonal antibodies (PAb) recognized proteins that were greater than 150 kDa in the culture supernatants of the MRP+EF- strains. These high molecular weight proteins were also detected by the anti-EF MAb, indicating that the 110 kDa EF and the > 150 kDa proteins share epitopes. In both the SDS-PAGE and Western blot, the phenotypes of the *S. suis* strains used as inocula were identical to the phenotypes of the isolates collected at the end of both experiments from tonsils and inflamed tissues of infected pigs.

Clinical signs of disease. In both experiments, rectal temperatures of all pigs inoculated with strains of the MRP+EF+ phenotype increased from day 2 pi onwards, with peaks at 41.8°C between days 4 and 9. Rectal temperatures of ten pigs inoculated with strains of the MRP+EF- phenotype were higher than 40°C for short periods of 24 to 96 h between days 2 and 22. Frequency of fever was highest in the MRP+EF+ groups (40%) (Table 3). The frequency of increased polymorphous leucocytes (PML) in blood was highest in the MRP+EF+ groups (Table 3). Analysis of variance was performed on the log of PML counts in blood samples of pigs inoculated with strains of the three phenotypes. Three days before inoculations no

significant differences were found between the geometric mean PML counts of th three groups. From day one pi onwards, the means of numbers of PML in blood samples of pigs inoculated with strains of the MRP+EF+ phenotype were significantly higher ($p<0.01$) than in either the MRP+EF- groups or the MRP-EF- groups. On day 20 pi, the means in the MRP+EF+ and MRP+EF- groups did not differ significantly from each other, but those means differed significantly ($p<0.01$) from the means in the MRP-EF- groups. Morbidity in pigs inoculated with strains of the MRP+EF+ phenotype was 100%. From day 2 onwards, non-specific signs of systemic disease, such as depression, recumbency, lack of appetite, and fever were observed. During the following days, pigs showed more specific signs of disease, such as ataxia, circular movements, opisthotonus, recumbency with paddling, and lameness. The frequency of specific signs of disease in the MRP+EF+ groups was 57% (Table 3). Nine pigs died in the course of the experiment, and three were killed in the terminal stages of disease. The mortality rate in these groups was thus 12/18 (67%). Nine pigs inoculated with strains of the MRP+EF- phenotype developed fever or granulocytosis or showed other nonspecific signs of disease, but did not show specific clinical signs, such as nervous disorders or lameness. Pigs in the MRP-EF- groups did not develop clinical signs of disease (Table 3).

TABLE 3. Frequency of three parameters of disease observed in pigs inoculated with *S. suis* type 2 (10 strains belonging to three phenotypes)

	Frequency[1] (%) of 3 parameters of disease			
S. suis phenotype	Fever > 40°C	PML in blood > 10^{10}/L	Clinical signs of disease specific[2]	non-specific[3]
MRP+EF+	40	78	57	21
MRP+EF-	5	16	0	5
MRP-EF-	0	3	0	0

1 Number of positive records / total number of records
2 Lameness and nervous disorders such as ataxia, circular movements, opisthotonus, and recumbency with paddling.
3 Depression, lack of appetite, and recumbency.

Pathologic findings are summarized in Table 4. Severe and frequent inflammations of the CNS, serosa, and joints were only detected in pigs inoculated with strains of the MRP+EF+ phenotype. Pneumonia and bronchitis were observed in various forms. Follicle formation in B cell areas and blast cell formation in T cell areas of the white pulp of the spleen - signs of active immune response - were more frequently observed in pigs inoculated with strains of the MRP+EF- phenotype (50%) than in

pigs inoculated with strains of the MRP-EF- phenotype (22%) or strains of the MRP+EF+ phenotype (11%) (Tabl 4). Some pigs inoculated with MRP+EF+ showed lymphocytolysis in the germinal centres, while the marginal zone surrounding the white pulp was inflamed, signs of acute septichaemia in young animals (42). Active follicles in tonsils were also more often seen in pigs inoculated with strains of the MRP+EF- or MRP-EF- phenotype.

TABLE 4. Pathologic lesions detected in various tissues of pigs inoculated with *S. suis* type 2 (10 strains of three phenotypes

Tissue and pathologic lesions	No. of pigs with pathologic lesions		
	phenotype MRP+EF+ (no. tested = 18)	phenotype MRP+EF- (no. tested = 12)	phenotype MRP-EF- (no. tested = 22)
CNS			
Meningitis[1]	12	0	0
Encephalitis[1]	10	1	0
Choroiditis	7	0	0
Malacia	5	0	0
Serosae/joints			
Peri-/epicarditis	11	1	1
Pleuritis	5	1	0
Peritonitis	14	6	0
Polyarthritis[2]	15	0	0
Lungs			
Cath. broncho-pneumonia	1	1	1
Fibrinous pneumonia	3	0	0
Interstitial pneumonia	7	5	5
Bronchitis/ Peribronchiolitis	2	2	3
Liver			
Periportal and/or intralobular foci	11	8	3
Spleen			
Active white pulp	2	6	5
Active red pulp	4	0	2
Tonsil			
Active follicles	3	9	12
Exudation in crypts	1	5	6

[1] Affecting cerebrum, cerebellum, pons, mesencephalon and medulla oblongata in various combinations.

[2] Affecting carpal, metacarpal, tarsal, metarsal, knee, elbow, shoulder and hip joints in various combinations.

Bacteriologic findings. From day 1 one pi to the end of the experiment, the streptococcal strains and *B. bronchiseptica* were isolated daily from naso-pharyngeal and fecal swab specimens of all pigs. A Bacillus species was also isolated from day six pi onwards from pigs inoculated with

strain 16 (experiment I) and from day 19 pi onwards from pigs inoculated with strain 24 (experiment II). Pigs in the other groups remained free from contaminating bacteria.

At necropsy, *S. suis* type 2 was mostly isolated from organs and tissues (CNS, serosae, and joints) that also showed pathologic changes (Table 5). *B. bronchiseptica* was only isolated from lungs and tonsils. Both *S. suis* and *B. bronchiseptica* were also isolated from the tonsils of all pigs.

TABLE 5. Isolation of streptococci from various tissues of pigs inoculated with *S. suis* type 2 (10 strains of three phenotypes).

Tissue	No. of pigs from which *S. suis* was isolated at necropsy phenotype MRP+EF+ (no. tested = 18)	phenotye MRP+EF- (no. tested = 12)	phenotype MRP-EF- (no. tested = 22)
CNS	14	0	0
Serosae	9	2	0
Joints	13	2	0
Lungs	6 (9)	0 (2)	2 (8)

[1] Numbers in parentheses indicate number of pigs from which *B. bronchiseptica* was also isolated.

EXAMPLE 6

Discrimination between Virulent and Nonvirulent *Streptococcus suis* type 2 Strains by Enzyme-Linked Immunosorbent Assay

MATERIALS AND METHODS

Bacteria. 179 strains of *S. suis* type 2 obtained from three sources were examined: from organs of diseased pigs in the course of routine diagnostic procedures, from tonsils of healthy pigs at slaughter, and from human patients suffering from *S. suis* type 2 infection. SDS-PAGE and Western blotting techniques were used in an earlier study to detect MRP and EF in culture supernatants, and on the basis of these results strains were categorized into three phenotypes: MRP+EF+, MRP+EF-, and MRP-EF- (Example 4). Also tested were 22 strains of *S. suis* serotypes 1 to 22 (*15*), 22 other streptococci, 20 bacterial strains of 15 different species, and one yeast (DLO-Central Veterinary Institute, Lelystad) (Table 6).

TABLE 6 *List of microorganisms*

Group	Microorganisms	Microorganisms
A	*Streptococcus pyogenes humanis*	Other bacterial species:
B	*Streptococcus agalactiae*	*Staphylococcus aureus*
C	*Streptococcus equi*	*Staphylococcus epidermidis*
	Streptococcus equisimilis porcine	*Staphylococcus hyicus*
	Streptococcus dysgalactiae	*Aerococcus viridans*
	Streptococcus zooepidemicus	*Actinomyces pyogenes*
D	*Enterococcus faecalis*	*Escherichia coli* (3x)
	Enterococcus faecium	*Klebsiella oxytoca*
	Enterococcus liquefaciens	*Klebsiella pneumoniae*
	Streptococcus bovis (2x)	*Micrococcus* strain 3551
	Streptococcus zymogenes	*Micrococcus luteus*
E	*Streptococcus* group E	*Pasteurella multocida* (4x)
G	*Streptococcus* group G (2X)	*Proteus vulgaris*
L	*Streptococcus* group L (2X)	*Salmonella typhimurium*
P	*Streptococcus* group P	*Serratia liquefaciens*
Q	*Streptococcus* group Q	
	Streptococcus milleri III	Yeast:
	Streptococcus sanguis	*Cryptococcus laurentii*
	Streptococcus uberis	

Culture conditions and antigen preparation. A 1 day old colony of the bacteria grown overnight on Columbia blood agar base (code CM 331, Oxoid Ltd.) containing 6% horse blood was inoculated into Todd-Hewitt broth (code CM 189, Oxoid). After overnight growth at 37°C, cultures were centrifuged at 4000 x g for 15 min. At 600 nm the optical densities of the 20 hour cultures were found to vary from 0.60 to 1.04. Some species had lower densities, these were *Bordetella bronchiseptica* (0.23), *Micrococcus* species (0.08 to 0.15), *Streptococcus equinus* (0.36), *Cryptococcus neoformans* (0.05). Twofold serial dilutions of untreated culture supernatants were used as test samples in the two DAS-ELISAs. Culture supernatant of *S. suis* type 2 strain D_{282} (MRP+EF+) was concentrated and partially purified by ultrafiltration (type PM30 filters, Amicon Cooperation). It was diluted in phosphate-buffered saline (PBS) (136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 2.79mM KH_2PO_4, pH 7.2), to a final protein concentration of 75 µg/ml. This product was used as coating antigen for the selection of different monoclonals in the direct competition ELISA and for screening hybridoma culture supernatants in the indirect ELISA.

Preparation of polyclonal and monoclonal antibodies. Rabbit (Ra) polyclonal antibodies (PAb) directed against MRP and EF (Ra K_{191}) and three different MAbs directed against EF were prepared as described in Example 4. MAbs that specifically recognize MRP were prepared essentially

the same as MAbs that recognize EF. Antigen production and immunization procedures in female BALB/c mice have been described (Example 4). Hybridoma cell lines were prepared as described (*52*). After 10 to 14 days, hybridoma culture supernatants were tested for antibodies against MRP in an indirect ELISA (see below). Hybridoma culture supernatants (diluted 1:2) were then tested on Western blots of culture supernatants of strain D-282 for antibodies directed against MRP. Bound MAb to the 136 kDa protein were visualized by using anti-mouse immunoglobulins conjugated to alkaline phosphatase and the substrate decribed below. Five supernatants were found positive, and the cells from these wells were cloned twice by limiting dilution in microtiter plates.

The five cell lines that were positive for anti-MRP antibodies and the three cell lines that were positive for anti-EF antibodies were used to produce ascites fluid in pristane-primed male BALB/c mice. MAbs directed against MRP and EF were purified from ascites fluid by ammonium sulphate precipitation (50% saturation) and dialysed against PBS. The five anti-MRP MAbs were designated: MRP_1 to MRP_5, the three anti-EF MABs were designated: EF_1 to EF_3. The immunoglobulin isotype of all MAbs was IgG_1 and was determined by double immunodiffusion with mouse isotype-specific antisera (Nordic) in gels of 1% agarose in PBS. The PAbs and MAbs were stored at -20°C.

Indirect ELISA for screening hybridoma culture supernatants. Polystyrene microtiter plates (Greiner, Nürtingen, Germany) were coated for 16 h at 37°C with the solution of concentrated and dialysed culture supernatant of strain D-282 (see above). They were then diluted in PBS, pH 7.2 (75 µg/ml protein). Twofold dilutions of hybridoma culture supernatants were added to the wells according to the procedure described by Van Zijderveld et al. (*51*). After the plates were washed, antimouse immunoglobulins (diluted 1:500) conjugated with horse radish peroxidase (HRPO, Nordic) were added. After incubation for 1 h at 37°C and five washings, the bound HRPO-antibody was then detected by the addition of substrate, 0.1% (w/v) solution of recrystallized 5-aminosalicylic acid (5-AS) (Merck) in 0.01 M phosphate buffer, pH 5.95, containing 0.01M sodium EDTA to which H_2O_2 had been added, immediately before use to an end concentration of 0.005% (wt/vol). After 2 h incubation at room temperature, the absorbance was measured at 450 nm with a Titertek Multiskan photometer (Flow Labs).

Direct competition ELISA. MAbs were selected with the direct competition ELISA and were used to develop the MRP and EF double antibody sandwich (DAS) ELISAs. Purified anti-MRP and anti-EF MAbs and rabbit PAbs were

conjugated to HRPO (Boehringer Mannheim, Germany) with th periodate method of Wilson and Nakane (*49*). Conjugated immunoglobulins were stored at -20°C in 50% glycerol. Conjugate solutions were made in PBS-Tw containing 5% fetal calf serum and 0.5% sodium chloride. 50 µl of non-conjugated anti-MRP MAbs in serial twofold dilutions (range 1:20 to 1:10,240) were added to the wells of polystyrene microtiter ELISA plates (Greiner) that had been coated with the culture supernatant of strain D_{282} that had been partially purified in PBS (75 µg/ml protein). The plates were then incubated for 30 min at 37°C. To allow the nonconjugated MAb to compete with the MAb conjugates, 50 ml of the optimal dilution of each of the five anti-MRP MAbs conjugated to HRPO were added. After incubation for 1 h at 37°C, plates were washed and the bound HRPO antibody was then detected by the addition of the substrate 5-AS H_2O_2 as described above. After 2 h incubation at room temperature, the absorbance was read. The titers of competition were expressed as the highest dilution showing an A_{450} of 50% of the mean absorbance of wells to which only conjugate was added. The epitope specificity of the three anti-EF MAbs was determined with a competition ELISA similar to the one described for the anti-MRP MAbs.

<u>SDS-PAGE and Western blotting techniques</u>. Culture supernatants of the 22 *S. suis* serotypes and the other microrganisms (Table 6) were separated by SDS-PAGE on 6% polyacrylamide. For Western blot analysis, the proteins were electroblotted onto nitrocellulose by the Multiphor II Nova Blot system according to the recommendations of the manufacturer (Pharmacia LKB). The blots were probed with a 1:300 dilution of mouse MAb. Bound MAbs were visualized with a 1:1000 dilution of anti-mouse immunoglobulins conjugated with alkaline phosphatase (Zymed).

RESULTS

<u>Direct competition ELISA</u>. The five anti-MRP clones and the three anti-EF clones were tested for competition. Some anti-MRP clones competed with each other. The five anti-MRP MAbs were directed against at least three different epitopes: the first was recognized by MRP_1 and MRP_2, the second by MRP_3, and the third by MRP_4 and MRP_5. Because all three anti-EF clones competed, they are probably directed against the same epitope.

<u>MRP double antibody sandwich ELISA</u>. In an MRP DAS-ELISA using MRP_3 as catching antibody and HRPO-MRP_1 as conjugate, each well of the polystyrene microtiter ELISA plates was coated with 100 µl containing 2.3 µg MRP_3 per well in 0.05 M carbonate buffer, pH 9.6. After adsorption for 16 h at 37°C, coated plates were used immediately or stored at -20°C.

Twofold serial dilutions of 100 µl culture supernatants, ranging from 1:1 to 1:128 in PBS containing 0.05% (wt/vol) Tween 80, of strains to be tested, were added to the wells. After 1 h incubation at 37°C, plates were washed five times with 0.05% Tween 80 in tap water, and 100 µl solution containing 2.2 µg of the HRPO-conjugated MRP_1 in PBS pH 7.2. was added to each well. Using checker-board titrations, the optimal dilution of catching antibody and conjugate was determined. After 1 h incubation at 37°C, the substrate 5-AS H_2O_2 was added as described above. Wells with an $A_{450} \geq 0.2$ were scored positive. To each plate a positive control was added, consisting of 100 µl of undiluted culture supernatant of the virulent *S. suis* type 2 strain 4005 (MRP+EF+). A negative control was also added, consisting of 100 µl of undiluted culture supernatant of the non-virulent strain T-15 (MRP-EF-) (*43*).

The MRP DAS-ELISA was used to test 179 strains of *S. suis* type 2 belonging to the three phenotypes MRP+EF+, MRP+EF-, and MRP-EF-, as was previously determined by SDS-PAGE and Western blot. Most strains scored in the ELISA the same as they did in the Western blot (Table 7). All MRP+EF+ strains were MRP-positive in the ELISA. One MRP+EF- strain scored false negative. Three of the MRP-EF- strains (6%) scored false positive. The sensitivity (TP/TP+FN) (TP = true positive, FN = false negative) of the MRP DAS-ELISA was 99% (130 out of 131 strains), the specificity (TN/TN+FP) (TN = true negative, FP = false positive) was 94% (45 out of 48 strains), and the predictive value (TP/TP+FP) was 98% (130 out of 133 strains). The MRP DAS-ELISA discriminated well between the MRP-positive and MRP-negative strains of *S. suis* type 2.

TABLE 7 Results of 179 strains of *S. suis* type 2 (three phenotypes) tested in the MRP and EF DAS-ELISAs.

	MRP DAS ELISA		EF DAS ELISA	
phenotype	No. strains +	No. strains -	No. strains +	No. strains -
MRP+EF+	92 (100%)	0	92 (100%)	0
MRP+EF-	38 (97%)	1 (3%)	0	39 (100%)
MRP-EF-	3 (6%)	45 (94%)	0	48 (100%)

Titration curves of culture supernatants of strains belonging to three phenotypes of *S. suis* type 2, after testing in the MRP DAS-ELISA, were recorded. The mean (± standard deviation) of the absorbances obtained from the undiluted culture supernatants of the 92 MRP+EF+ isolates was

1.2259 (± 0.1165), the mean absorbance of the 39 MRP+EF- isolates was 1.2129 (± 0.2076), and the mean absorbance of the 48 MRP-EF- isolates was 0.1180 (± 0.2546). Therefore plates can be read visually instead of having to be measured photometrically to discriminate MRP-positive strains (phenotypes MRP+EF+ or MRP+EF-) from MRP-negative strains (phenotype MRP-EF-).

Culture supernatants of 18 of the 21 reference *S. suis* strains of other serotypes had absorbances lower than 0.2. Three serotypes were positive and had the following absorbance values: undiluted culture supernatant of serotype 3 had A_{450} = 0.731; culture supernatant of serotype 5 had A_{450} = 0.587, and culture supernatant of serotype 15 (former Lancefield group T) had A_{450} = 0.516. These serotypes were also positive in the Western blot; MRP_3 apparently recognized proteins of higher molecular weight than 150 kDa in the culture supernatants of these serotypes. Absorbances of all other microorganisms listed in Table 6 were < 0.2.

EF Double Antibody Sandwich ELISA. In a DAS ELISA that recognizes a specific antigen in the test sample, two different MAbs were used, one as catching antibody and the other as conjugate, and each recognizing different epitopes on the antigen, as was done for the MRP DAS-ELISA. In the Western blot the EF MAbs recognize a high molecular form of EF (> 150 kDa) in the culture supernatants of all strains belonging to the MRP+EF- phenotype (Example 4). Therefore it is unlikely that an ELISA with EF_2 as catching antibody can discriminate between MRP+EF+ and MRP+EF- strains. Moreover, because the three EF MABs blocked each other, we had to use EF_2 as catching antibody and the polyclonal rabbit serum (K_{191}) as conjugate. Some ELISAs were tested using EF_1 as catching antibody and EF_2 or EF_3 as conjugates, and indeed these MAbs blocked each other completely.

The procedure of the EF DAS-ELISA was essentially as that described for the MRP DAS-ELISA. Each well of the microtiter ELISA plates was coated with 100 ml containing 3.3 µg of EF_2 in 0.05M carbonate buffer, pH 9.6. After adsorption, coated plates were used immediately or stored at -20°C. Twofold serial dilutions of 100 µl culture supernatants ranging from 1:1 to 1:128 were used. After incubation and washings, 100 µl containing 2.7 µg polyclonal Ra K_{191} HRPO conjugate in PBS, pH 7.2, was added to each well. After 1 h incubation at 37°C, the plates were developed with substrate 5-AS H_2O_2 as described above. Wells with an $A_{450} \geq 0.4$ were scored positive. The same controls as mentioned above were used on each plate.

The 179 *S. suis* type 2 strains with a predetermined protein profile were

tested in the EF DAS-ELISA. Surprisingly, none of the 39 MRP+EF- strains scored positive in this ELISA, whereas all 92 MRP+EF+ strains did (Table 7). All 48 MRP-EF- strains were negative in the EF DAS-ELISA. Since no other false positive or false negative results were detected, the EF DAS-ELISA apparently discriminated reliably between the high and the low molecular form of EF, and hence between *S. suis* type 2 strains belonging to the MRP+EF+ and MRP+EF- phenotypes.

Since the direct competition ELISA had shown that the three anti-EF MAbs blocked each other, MAb EF_2 was used as catching antibody and the polyclonal Ra K_{191} serum as conjugate. Streptococcus suis type 2 strains belonging to the MRP+EF- phenotype, however, produce a high-molecular weight (>150 kDa) form of EF (example 4). Because MAb EF_2 does not discriminate between the 110-kDa EF and this high-molecular weight form in the Western blot, it was unlikely to do so in the EF DAS-ELISA. Surprisingly Mab EF_2 captured the 110-kDa EF in the culture supernatant of all MRP+EF+ strains but apparently not the higher-molecular weight form in the MRP+EF- strains (Table 7). Some MRP-EF- strains gave signals between 0.2 and 0.4, which were still lower than 50% of the maximal absorbance values and thus not high enough to be interpreted as positive. Treating the culture supernatants with SDS before blotting may uncover epitopes of the higher-molecular weight form of EF that are not accessible to the EF_2 MAb in its undenaturated form. Because all MRP-EF- strains and other S. suis serotypes showed no false negative or false positive reactions in this ELISA, the sensitivity and specificity of the tests were considered to be 100%.

Titration curves of culture supernatants of strains belonging to three phenotypes of *S. suis* type 2 were recorded after testing in the EF DAS-ELISA. The mean (± standard deviation) of the absorbances obtained from the undiluted culture supernatants of the 93 MRP+EF+ strains was 0.8204 (± 0.149), the mean absorbance of the 39 MRP+EF- strains was 0.1551 (± 0.046), and the mean absorbance of the 48 MRP-EF- strains was 0.1061 (± 0.0371). Thus, as for the MRP DAS-ELISA, plates can be read visually to discriminate between EF-positive strains (phenotype MRP+EF+) and EF-negative strains (phenotypes MRP+EF- or MRP-EF-).

None of the 21 reference *S. suis* strains with a serotype other than type 2 were EF-positive in the ELISA. Some other bacterial species had positive absorbance values: *Streptococcus* Lancefield group G (A_{450} = 0.445), group L (A_{450} = 0.348), *Streptococcus equi* (A_{450} = 0.671), and *Staphylococcus aureus* (A_{450} = 0.718).

EXAMPLE 7

Differentiation between pathogenic and non-pathogenic strains of *Streptococcus suis* type 2 by using polymerase chain reaction (PCR).

MATERIALS AND METHODS.

Bacteria and growth conditions. Thirteen strains of *S. suis* type 2 were selected to examine whether the Polymerase Chain Reaction (PCR) method (*36*) could be useful to differentiate between the three phenotypes of *S. suis* type 2. Pathogenicity and the expression of the MRP and EF proteins of these strains were determined in Examples 4 and 5. Strains were grown overnight at 37°C on Columbia blood agar base (code CM 331, Oxoid) containing 6% horse blood. *S. suis* type 2 colonies were inoculated in 10 ml Todd-Hewitt broth (code CM 189, Oxoid), and grown overnight at 37°C.

DNA Isolations. DNA of overnight grown cultures was isolated as described by Maniatis et. al (*28*). DNA was diluted to 10 ng/µl in distilled water before use in the PCR.

Clinical specimens. Nose swabs and tonsillar tissues were obtained post mortem from sows at slaughter. Nose swabs were inoculated on blood plates. *S. suis* type 2 strains were isolated from tonsils as described before (*27*).

Sample preparation. Clinical specimens for the PCR were prepared by the method described by Boom et. al (*4*), with some minor modifications: The specimens were added to 900 µl L6 lysis buffer plus 40 µl diatom earth solution in an Eppendorf tube [L6 buffer is 100 ml 0.1 M TRIS HCl (pH 6.4) plus 120 g guanidine (iso)thiocyanate (GuSCN, Fluka cat nr. 50990) plus 22 ml 0.2 M EDTA (pH 8.0) plus 2.6 g Triton X-100. Diatom earth solution is 10 g Diatom earth (Janssen Chimica Cat. nr. 17.346.80) in 50 ml distilled water plus 500 µl 32% (w/v) HCl]. The clinical specimens were incubated overnight in L6 buffer in the dark at room temperature. 150 µl of the solution was pipetted in wells of microtiter plates containing Durapore membranes (Multiscreen MAHV N45, Millipore). The microtiter plate was put on the vacuum manifold (MAVM 09600, Millipore), and the samples were washed 5 times with 200 µl L2 washing solution (L2 buffer is 100 ml 0.1 M Tris-HCl (pH 6.4) plus 120 g GuSCN), 5 times with 200 µl 70% ethanol, and once with 200 µl aceton. The filters were not allowed to run dry between the wash steps. The bottom of the microtiter plate was dried on a tissue and the samples were dried completely for 15 minutes at 56°C. 75 µl PCR buffer (see below) was added to the individual wells. Th plate was incubated for 15 minutes at 56°C. The microtiter plate was again put on the vacuum manifold, with a standard microtiter

plate (Micronic) beneath the Durapore plate. Vacuum was applied, and the PCR buff r, containing the DNA was collected in the lower microtiter plate, whereas the diatom earth remained on the Durapore filters.

PCR assay. The PCR contained 10 ng purified DNA or 25 µl clinical specimen in a total volume of 50 µl. The reaction mixtures contained 10 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.2 mM of each of the four deoxynucleotide triphosphates, 1 µM of each of the four primers and 0.5 U of Amplitaq polymerase (Perkin Elmer Cetus, Norwalk, Conn.), and was overlaid with 2 drops of paraffine oil. DNA amplification was carried out in a Perkin Elmer Thermal Cycler for 25 or 40 cycles: 1 minute 94°C, 1 minute 55°C, and 2 minutes 72°C. Ten to 20 µl of the amplified DNA was analysed on a 1.5% agarose gel, that contained ethidium bromide.

PCR primers. The sequences of the oligonucleotides used in the PCR were: p15: 1403-1425: 5'- GGT ATA CCT TGC TGG TAC CGT TC -3', p16: 1914-1934: 5'- AGT CTC TAC AGC TGT AGC TGG -3', p-34: 2890-2908: 5'- GTT GAA AAC AAA GCA TTC G -3', and p-35: 3229-3249: 5'- CTT CGA CAA AAT GTC AGA TTC -3'. The oligonucleotides p-15 and p-16 correspond to the indicated positions in the *S. suis* type 2 *mrp* gene (Example 3, Fig. 2). The oligonucleotides p-34 and p-35 correspond to the indicated positions and in the *S. suis* type 2 *ef** gene (Example 2, Fig. 1B). Primers were synthesized on an Applied Biosystem synthesizer type 381A following the manufacturers protocol.

RESULTS

Specificity of PCR. Within the *mrp* and *ef** genes (cf. Examples 3 and 2), two regions (designated as m-VI and e-V) were determined that could be used to differentiate between the three phenotypes of *S. suis* type 2 strains (see also Example 8). Primers based on the m-VI region (p-15 and p-16), and the e-V region (p-34 and p-35) were used in a PCR. The primers p-15 and p-16 amplified a 532 bp fragment in the m-VI region. The primers p-34 and p-35 amplified a 360 bp fragment in the e-V region. Chromosomal DNA of 4 MRP^+EF^+, 4 MRP^+EF^* and 5 MRP^-EF^- strains was used in a PCR with these primers (see Fig. 15). After 25 cycli the amplified fragments were analysed on an agarose gel. A 532 bp fragment was amplified from DNA of MRP^+EF^+ strains. A 532 bp fragment as well as a 360 bp fragment were amplified from DNA of MRP^+EF^* strains. In contrast, neither the 532 bp nor the 360 bp fragment was amplified from DNA of MRP^-EF^- strains. These data show that this PCR can be used to differentiate between the three phenotypes of *S. suis* type 2.

The phenotypes of 82 strains of *S. suis* type 2, isolated from the tonsils of 37 healthy sows at slaughter, were determined by Western blotting (Example 4), ELISA (Example 6), hybridization experiments with DNA probes m-VI and e-V (Example 8), and by PCR. 79 strains, isolated from 36 of the 37 sows were classified identical by the four methods (96.3%). 3 strains, isolated from one sow, were classified as $MRP^{+}EF^{*}$ by the PCR and DNA hybridization experiments and as $MRP^{-}EF^{-}$ by Western blotting and ELISA. These results indicate that the PCR is a useful alternative to determine the phenotype of a *S. suis* type 2 strain.

Sensitivity of PCR. Purified chromosomal DNA of a $MRP^{+}EF^{*}$ *S. suis* type 2 strain was diluted in distilled water and used directly in the PCR. After 40 cycli of PCR, 25 fg DNA was detected. This indicates that DNA of 14 cells, after amplification by PCR, could be detected on an agarose gel, based on data that a Streptococcal cel contains about 1.75 fg DNA (35). The sensitivity of the PCR on whole cells was determined. Therefore, $MRP^{+}EF^{*}$ cells were diluted in phosfate buffered saline (PBS (pH 7.2); 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 2.8 mM KH_2PO_4) and prepared for PCR as described above. Amplified fragments could still be detected in samples that contained about 50 cells prior to the PCR (40 cycli).

The PCR can be used directly on clinical material. Serial dilutions of *S. suis* type 2 cells were added to nose swabs. It was found that amplified fragments can still be detected in samples that contain about 50 cells prior to the PCR.

EXAMPLE 8

Differentiation between pathogenic and non-pathogenic strains of *S. suis* type 2 using DNA probes.

MATERIALS AND METHODS

Bacteria. Thirteen strains of *S. suis* type 2 (4 $MRP^{+}EF^{+}$ strains, 4 $MRP^{+}EF^{*}$ strains and 5 $MRP^{-}EF^{-}$ strains) were selected to examine whether regions of the *mrp*, *ef*, and *ef** genes could be useful to differentiate between the three phenotypes of *S. suis* type 2. Except for strain 16, pathogenicity of these strains was tested in an infection experiment of piglets (Example 5).

170 strains of *S. suis* type 2 were obtained from three sources: From organs of diseased pigs (103 strains), from tonsils of healthy pigs at slaughter (40 strains) and from human patients (27 strains). Reference strains of *S. suis* serotypes 1 to 22 (15), 21 other *Streptococci* species and 45 other bacterial strains (38 different species, DLO Central

Veterinary Institute, Tabl 8) were used to test the specificity of the *mrp* and *ef* probes.

Media. *E. coli* JM101 strains were grown in LB broth (*30*). Ampicillin was added as needed to a final concentration of 50 µg/ml. All other bacterial strains were grown overnight at 37°C on Columbia blood agar base (code CM 331, Oxoid) containing 6% horse blood. Overnight grown colonies were incubated in 10 ml Todd-Hewitt broth (code CM 189, Oxoid), and grown overnight at 37°C.

DNA isolations and manipulations. Chromosomal DNA isolations and routine DNA techniques were performed as described by Maniatis et al (*28*). Crude lysates were made as follows: overnight grown cultures were centrifuged at 4000 x g for 10 minutes, and the pellet fraction was resuspended in 500 to 1000 µl TEG-lysozym buffer (25 mM TRIS.Cl pH 8.0, 10 mM EDTA, 50 mM glucose and 1 mg/ml lysozym). After 30 minutes at 25°C, the samples were used in the dot-blot assay.

Probes. The plasmids pMR11, pEF2-19 and pEF17-7 (cf. Examples 1, 2, 3) were used to generate subclones into pKUN19 (*24*). Fragments of appropriate subclones were isolated from preparative agarose gels with the gene-clean kit (Bio 101 Inc., La Jolla, USA). Purified fragments were subsequently labeled with α-^{32}P dCTP (3000 Ci/mMol, Amersham) with the random primed labeling kit (Boehringer GmbH) following the manufacturers protocol and used as probes.

Southern hybridizations. Chromosomal DNA of the 13 selected *S. suis* 2 strains (1 µg DNA) was spotted on Gene-screen nylon membrane (New-England Nuclear Corp., Boston, USA). The membranes were incubated with the ^{32}P-labeled *mrp* and *ef* probes as recommended by the manufacturer. After overnight hybridization, the filters were washed twice with 2 x SSC for 5 minutes at room temperature, and twice with 0.1 x SSC plus 0.5% (SDS) for 30 minutes at 65°C (1 x SSC = 0.15 M NaCl plus 0.015 M Sodium Citrate). For the group of 170 *S. suis* 2 strains, the 22 reference strains of *S. suis* type 1 to 22, and the group of other *Streptococci* and other bacteria, 20 µl of a DNA or crude lysate sample was dotted on Zeta probe nylon membrane (Biorad) with a dot blot apparatus (Bethesda Research Laboratories).

The membranes were incubated with the ^{32}P-labeled *mrp* and *ef* probes as recommended by the manufacturer. After overnight hybridization, the membranes were washed twice in 40 mM Na phosphate buffer, pH 7.2 plus 5% SDS plus 1 mM EDTA for 30 minutes at 65°C and twice in 40 mM Na phosphate buffer pH 7.2 plus 1% SDS plus 1 mM EDTA for 30 minutes at 65°C. All

(pre)hybridizations were carried out in a hybridization oven (Hybaid).

RESULTS

Mrp probes. Chromosomal DNA of the 3 phenotypes of *S. suis* type 2 was hybridized to different regions of the *mrp* gene. Six different *mrp* probes were used (schematically shown in Fig. 14a). The EcoRI-SnaBI fragment, m-I, contained the entire *mrp* encoding region. The m-II, m-III, m-IV and m-V probes contained different regions of the *mrp* gene (see Fig. 16). The MRP$^+$EF$^+$ and the MRP$^+$EF* strains strongly hybridized with all *mrp* probes. In addition, the m-I, m-II, m-IV and m-V probes strongly hybridized with 4 of the 5 MRP$^-$EF$^-$ strains. One MRP$^-$EF$^-$ strain (strain 25) did not hybridize with any of the *mrp* probes. These data indicate that 4 MRP$^-$EF$^-$ strains contained large regions homologous to the *mrp* gene of strain D282, whereas strain 25 lacked the entire *mrp* gene. These 4 MRP$^-$EF$^-$ strains, however, hybridized only weakly with probe m-III, indicating that only a small part of probe m-III was homologous to their DNA. A probe m-VI was constructed by removing 385 bp at the 5', and 325 bp at the 3' ends of probe m-III. The 5 MRP$^-$EF$^-$ strains did not hybridize at all with probe m-VI, indicating that these strains lacked the region homologous to the m-VI probe. Therefore, probe m-VI can be used to differentiate between MRP$^+$ and MRP$^-$ strains.

Ef and ef* probes. Chromosomal DNA of the 3 phenotypes *S. suis* type 2 was hybridized to different regions of the *ef* gene. Four different *ef* probes (schematically shown in Fig. 14b) were used. All MRP$^+$EF$^+$ and MRP$^+$EF* strains and 1 MRP$^-$EF$^-$ strain hybridized with all *ef* probes. In contrast, 4 MRP$^-$EF$^-$ strains did not hybridize with any of the *ef* probes. These data indicate that most of these MRP$^-$EF$^-$ strains lacked the entire region homologous to the *ef* gene, whereas 1 MRP$^-$EF$^-$ strain seemed to contain the entire region homologous to the *ef* gene. Therefore, the probes e-I to e-IV could not been used to differentiate between the 3 phenotypes.

Since the gene encoding the EF* proteins contain a DNA fragment which is absent in the gene encoding the EF protein, part of this extra DNA was selected as a probe (Fig. 14c, probe e-V). Probe e-V hybridized with all MRP$^+$EF* strains. On the contrary, none of the MRP$^+$EF$^+$ and MRP$^-$EF$^-$ strains hybridized with the e-V probe. These data suggest that the MRP$^+$EF$^+$ and MRP$^-$EF$^-$ strains lacked the region homologous to e-V. Probe e-V is thus specific for MRP$^+$EF* strains.

Therefore, if m-VI and e-V are used in complementary hybridization studies, a differentiation between the three phenotypes of *S. suis* type 2 will be possible. If *S. suis* type 2 strains hybridize with probe m-VI

and e-V, th se strains belong to the MRP$^+$EF* phenotype. If *S. suis* type 2 strains hybridize with m-VI but not with e-V, these strains belong to the MRP$^+$EF$^+$ phenotype, and finally if strains do not hybridize with m-VI and e-V, these strains belong to the MRP$^-$EF$^-$ phenotype.

The *mrp*, *ef* and *ef** probes were tested on 170 other strains of *S. suis* type 2. 88 strains had a MRP$^+$EF$^+$ phenotype, 37 strains a MRP$^+$EF* phenotype and 45 strains had a MRP$^-$EF$^-$ phenotype. In accord with the data presented above, all MRP$^+$EF$^+$ strains hybridized with the probes m-I to m-VI and e-I to e-IV, but none hybridized with probe e-V. Moreover, all the 37 MRP$^+$EF* strains hybridized with all the probes. Only two of the 45 MRP$^-$EF$^-$ strains, however, hybridized with probe m-VI and e-V and would therefore wrongly be classified as MRP$^+$EF* strains. Therefore, by using m-VI and e-V, the phenotype of a *S. suis* type 2 strain can be predicted with a very high probability (168/170; 98.8%).

<u>Specificity of the m-VI and e-V probes</u>. DNA of the reference strains of *S. suis* serotype 1 to serotype 22 was tested for hybridization with probes m-VI and e-V. It was found that *S. suis* type 2 (strain 735), 4, 5 and 14 hybridized with the m-VI probe and that type 1/2, 2, 4, 5, 6, 14 and 15 hybridized with the e-V probe. These data suggest that the *mrp* and *ef* genes are not specific for *S. suis* type 2, but that homologous sequences are present in several serotypes. Based on these data, serotypes 2, 4, 5 and 14 would be classified as MRP$^+$EF* strains, whereas serotypes 1/2, 6 and 15 would be classified as MRP$^-$EF$^-$ strains.

Chromosomal DNA from swine pathogens and several common bacteria was tested with the probes m-I, m-VI, e-III and e-V. The species tested are listed in Table 8. Although some species hybridized with probe m-I (*Escherichia coli*, *Klebsiella oxytoca*, *K. pneumoniae* and *Salmonella typhimurium*), none hybridized with the probes m-VI, e-III and e-V. These data show that although in some species parts of the *mrp* gene are found, the probes m-VI and e-V are specific for *S. suis*. Hence, the probes m-VI and e-V have potential diagnostic value.

TABLE 8 List of other species on which the probes were tested for specificity.

<u>Streptococcus species</u>	
S. agalactiae	*S. equi*
S. equisimilis porcine	*S. zooepidemicus*
S. dysgalactiae	*Enterococcus faecalis*
E. liquefaciens	*E. zymogenes*
E. faecium	*S. group E*
S. milleri III	*S. bovis*
S. pyogenes humanis	*S. uburis*
S. animale G	*S. group G*
S. group L biotype I	*S. group L biotype II*
S. group P	*S. group Q*
S. sanguis	
<u>Other Bacteria</u>	
Actinobacillus pleuropneumoniae	
Actinobacillus suis	*Actinobacillus viridans*
Actinomyces pyogenes	*Aeromonas hydrophila*
Bacillus cereus	*Bacillus licheniformis*
Bacillus subtilis	*Bordetella bronchiseptica*
Brucella suis biotype I	*Brucella suis biotype II*
Campylobacter coli	*Campylobacter faecalis*
Campylobacter jejuni	*Candida albicans*
Clostridium perfringens A non-toxic	
Clostridium perfringens A toxic	
Escherichia coli	*Erysipelothrix rhusiopathiae*
Haemophilus parasuis	*Klebsiella oxytoca*
Klebsiella pneumoniae	*Listeria monocytogenes*
Micrococcus strain 3551	*Micrococcus luteus*
Mycobacterium avium serovar2	*Mycoplasma hyopneumoniae*
Mycoplasma hyorhinis	*Mycoplasma hyosynoviae*
Pseudomonas aeruginosa	*Pasteurella multocida*
Pasteurella vulgaris	*Salmonella typhimurium*
Serratia liquefaciens	*Staphylococcus aureus*
Staphylococcus epidermidis	*Staphylococcus hyicus hyicus*
Yersinia enterocolitica	

<u>LITERATURE REFERENCES</u>

1. Arends, J.P., and H.C. Zanen. 1984. Proc. 9th Lancefield Int. Symp. Streptococci Streptococcal Dis., p. 343-344.

2. Arends, J.P., and H.C. Zanen. 1988. Meningitis caused by *Streptoccus suis* in humans. Rev. Infect. Dis., 10: 131-137.

3. Arends, J.P., N. Hartwig, M. Rudolphy, and H.C. Zanen. 1984. Carrier rate of *Streptococcus suis* capsular type 2 in palatine tonsils of slaughtered pigs. J. Clin. Microbiol. 20: 945-947.

4. Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.W. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28: 495-503.

5. Breton, J., W.R. Mitchell, and S. Rosendal. 1986. *Streptococcus suis* in slaughter pigs and abbatoir workers. Can. J. Vet. Res., 50: 338-341.

6. Clifton-Hadley, F.A. 1983. *Streptococcus suis* type 2 infections. Br. Vet. J. 139: 1-5.

7. Clifton-Hadley, F.A., T.J.L. Alexander, M.R. Enright, and J. Guise. 1984. Monitoring herds for *Streptococcus suis* type 2 by sampling tonsils of slaughter pigs. Vet. Rec. 115: 562-564.

8. Clifton-Hadley, F.A., T.J.L. Alexander, I. Upton, and W.P.H. Duffus. 1984. Further studies on the subclinical carrier state of *Streptococcus suis* type 2 in pigs. Vet. Rec., 114: 513-518.

9. Driessen, A.M.J., W. de Vrij, and W.N. Konings. 1985. Incorporation of beef heart cytochrome c oxidase as a proton-motive-force generating mechanism in bacterial membrane vesicles. Proc. Natl. Acad. Sci. USA 82: 7555-7559.

10. Fahnestock, S.R., P. Alexander, J. Nagle and D. Filpula. 1987. Gene for an immunoglobulin-binding protein from a group G *Streptococcus*. J. Bacteriol. 167: 870-880.

11. Ferretti, J.J., R.R.B. Russell, and M.L. Dao. 1989. Sequence analysis of the wall-associated protein precursor of *Streptococcus mutans* antigen A. Mol. Microbiol. 3: 469-478.

12. Fischetti, V. A., V. Pancholi, and O. Schneewind. 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. Mol. Microbiol. 4: 1603-1605.

13. Frithz, E., L-O. Heden and G. Lindahl. 1989. Extended sequence homology between IgA receptor and M proteins in *Streptococcus pyogenes*. Mol. Microbiol. 3: 1111-1119.

14. Gogolewski, R.P., Cook, R.W. and O'Connel, C.J., 1990. *Streptococcus suis* serotypes associated with disease in weaned pigs. Austr. Vet. J., 67: 202-204.

15. Gottschalk, M., R. Higgins, M. Jaques, K.R. Mittal, and J. Henrichsen. 1989. Description of 14 new capsular types *Streptococcus suis*. J. Clin. Microbiol., 27: 2633-2636.

16. Guss, B., M. Uhlen, B. Nilsson, M. Lindberg, J. Sjöquist, and J. Sjödahl. 1984. Region X, the cell-wall-attachment part of staphylococcal protein A. Eur. J. Biochem. 138: 413-420.

17. Hager, P.W., and J.C. Rabinowitz. 1985. Translational specificity in *Bacillus subtilis*. In: The Molecular Biology of the Bacilli. Dubnau, D. A. (ed.). New York, Academic Press, pp 1-32.

18. Ham-Hoffies, A.M., L.A.M.G. van Leengoed, and A. Ho gendoorn. 1986. Calibration of a conducting counter in determining haematological values in blood samples of various animals. Proc. IVth Int. Symp. of Vet. Lab. Diagn. Amsterdam. p. 692-693.

19. Higgins, R., M. Gottschalk, K.R. Mittal, and M. Beaudoin. 1989. *Streptococcus suis* infections in swine. A sixteen month study. Can. J. Vet. Res., 54: 170-173.

20. Hollingshead, S.K., V.A. Fischetti, and J.R. Scott. 1987. The complete nucleotide sequence of type 6M protein of the group A *Streptococcus:* repetitive structure and membrane anchor. J. Biol. Chem. 261: 1677-1686.

21. Hommez, J., L.A. Devriese, J. Henrichsen, and F. Castryck. 1986. Identification and characterisation of *Streptococcus suis*. Vet. Microb., 16: 349-355.

22. Jaarsveld, B.C., E. van Kregten, R.G. van Kesteren, M. Rozenberg-Arska, and A.K.M. Bartelink. 1990. Fulminante sepsis door *Streptococcus suis*. Ned. Tijdschr. Geneesk., 134: 1462-1464.

23. Kamp, E.M., and T.G. Kimman. 1988. Induction of nasal turbinate atrophy in germ-free pigs, using *Pasteurella multocida* as well as bacterium-free crude and purified dermonecrotic toxin of *P. multocida*. Am. J. Vet. Res. 49: 1844-1849.

24. Konings, R.N.H., E.J.M. Verhoeven, and B.P.H. Peeters. 1987. pKUN vectors for the separate production of both DNA strands of recombinant plasmids. Methods Enzymol. 153: 12-34.

25. Kyte, J., and R.F. Doolittle. 1982. A simple method for displaying the hydrophatic character of a protein. J. Mol. Biol. 157: 105-132.

26. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of T4. Nature 227: 680-685.

27. Leengoed, L.A.M.G. van, U. Vecht, and E.R.M. Verheyen. 1987. *Streptococcus suis* type 2 infections in pigs in the Netherlands (part two). Vet. Quart. 9: 111-117.

28. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Second edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. New York.

29. Messing, J. 1979. A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. Recombinant DNA Technical Bulletin. NIH Publication no 79-99, 2, no 2, 43-48.

30. Miller, J. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor NY.

31. Moreau, A., R. Higgins, M. Bigras-Poulin, and M. Nadeau. 1989. Rapid detection of *Streptococcus suis* serotype 2 in weaned pigs. Am. J. Vet. Res., 50: 1667-1671.

32. Morissey, J.H. 1981. Silver stains for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117: 307-310.

33. Murray, N.E., W.J. Brammer, and K. Murray. 1977. Lamboid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. 150: 53-58.

34. Platt, T. 1986. Transcription termination and the regulation of gene expression. Annu. Rev. Biochem. 55: 339-372.

35. Pozzi, G., M.R. Oggioni, and A. Tomasz. 1989. DNA probe for identification of *Streptococcus pneumoniae*. J. Clin. Microbiol. 27: 370-372.

36. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, D.B. Mullis, and H.A. Ehrlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science 239: 487-491.

37. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74: 5463-5467.

38. Schneewind, O., K. F. Jones, and V. A. Fischetti. 1990. Sequence and structural characteristics of the trypsin-resistant T6 surface protein of group A Streptococci. J. Bacteriol. 172: 3310-3317.

39. Signas, C., G. Raucci, K. Jonsson, P-.E. Lindgren, G. M. Anantharamaiah, M. Hook, and M. Lindberg. 1989. Nucleotide sequence of the gene for a fibronectine-binding protein from *Staphylococcus aureus*: Use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. 16: 699-703.

40. Tinoco, Jr. I., P.N. Borer, B. Dengler, M.D. Devine, O.C. Uhlenbeck, D.M. Crothers, and J. Gralla. 1973. Improved estimation of secondary structure in ribonucleic acids. Nature 246: 40-41.

41. Uhlen, M., B. Guss, B. Nilsson, S. Gatenbeck, L. Philipson and M. Lindberg. 1984. Complete sequence of the Staphylococcal gene encoding protein A. J. Biol. Chem.259: 1695-1702.

42. Valli, V.E.O. 1985. The Hematopoietic System-Spleen and Hemolymph Nodes, p. 194-200. In: K.V.F. Jubb, P.C. Kennedy and N. Palmer, Pathology of Domestic Animals. Volume 3. 3rd ed. Academic Press, Inc. Orlando Florida.

43. Vecht, U., J.P. Arends, E.J. van d r Molen, and L.A.M.G. van Leengoed. 1989. Differences in virulence between two strains of *Streptococcus suis* type 2 after experimentally induced infection of newborn germ-free pigs. Am. J. Vet. Res., 50: 1037-1043.

44. Vecht, U., L.A.M.G. van Leengoed, and E.R.M. Verheyen. 1985. *Streptococcus suis* infections in pigs in The Netherlands (part one). Vet. Quart. 7: 315-321.

45. Von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14: 4683-4690.

46. Vos, P., G. Simons, R.J. Siezen, and W. M. de Vos. 1989. Primary structure and organization of the gene for a procaryotic cell envelope-located serine proteinase. J. Biol. Chem. 264: 13579-13585.

47. Vossen, J.M.B.M. van der, J. Kok, and G. Venema. 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 50: 540-542.

48. Williams, A.E. and W.F. Blakemore. 1990. Pathogenesis of Meningitis Caused by Streptococcus suis type 2. J. Infect. Dis. 162: 474-481.

49. Wilson, B. and Nakane, P.K., 1978. Recent developments in the periodate method of conjugating horse-radish peroxidase (HRPO) to antibodies. In: W. Knapp, K. Holubar, and G. Wicks (Editors), Immunofluorescence and related staining techniques. Biomedical Press, Elsevier, Amsterdam, pp 215-224.

50. Windsor, R.S. 1977. Meningitis in pigs caused by *Streptococcus suis* type 2. Vet. Rec. 101: 378-379.

51. Zijderveld, F.G. van, Westenbrink, F., Anakotta, J., Brouwers, R.A.M. and van Zijderveld, A.M., 1989. Characterization of the F41 Fimbrial Antigen of Enterotoxigenic *Escherichia coli* by Using Monoclonal Antibodies. Inf. and Imm. 57: 1192-1199.

CLAIMS

1. Use of a DNA sequence from a gene encoding a virulence characteristic of *Streptococcus suis*, for the diagnosis of, or for the protection against an infection by *S. suis* in mammals including man.

2. A DNA sequence of the gene encoding a 90,000 - 120,000 dalton polypeptide in virulent strains of *Streptococcus suis* and encoding a polypeptide having a higher molecular weight than the former polypeptide and substantially comprising the former polypeptide in less virulent strains of *Streptococcus suis*, which sequence or which gene has the nucleotide sequences according to figures 1A and 1B for *S. suis* serotype 2, strain D-282, or a corresponding sequence or a partial sequence thereof.

3. A DNA sequence of the gene encoding a 135,000 - 136,000 dalton polypeptide (Muramidase Released Protein) which is a virulence characteristic of *Streptococcus suis*, which sequence or which gene has the nucleotide sequence according to figure 2 for *S. suis* serotype 2, strain D-282, or a corresponding sequence or a partial sequence thereof.

4. A DNA sequence according to claim 2, encoding a part of the higher molecular weight polypeptide which part does not correspond to the 90,000 - 120,000 dalton polypeptide, or a partial sequence thereof.

5. A DNA sequence according to claim 2, which is a partial sequence containing at least 10 nucleotides, preferably at least 15 nucleotides, from the sequence 2890-3306 of Figure 1B.

6. A DNA sequence according to claim 3, which a partial sequence containing at least 10 nucleotides, preferably at least 15 nucleotides, from the sequence 1100-1934 of Figure 2.

7. A recombinant polynucleotide, comprising a sequence according to any one of claims 2-6 in the presence of a regulatory sequence.

8. A polynucleotide probe for the diagnosis of an infection by *Streptococcus suis*, comprising a sequence according to any one of claims 2-6.

9. A polypeptide encoded by, or obtained by expression of a sequence according to any one of claims 2-7.

10. An antibody raised against a polypeptide according to claim 9.

11. A method for detecting an infection by a pathogenic strain of *Streptococcus suis*, characterised by using at least one probe according to claim 8.

12. A method according to claim 11, wherein at least one probe having a sequence according to claim 4 or 5 is used.

13. A method for detecting an infection by a pathogenic strain of *Streptococcus suis*, characterised by using at least one polypeptide according to claim 9.

14. A method for detecting an infection by a pathogenic strain of *Streptococcus suis*, characterised by using at least one antibody according to claim 10.

15. A diagnostic kit for the detection of an infection by a pathogenic strain of *Streptococcus suis*, characterised by containing at least one probe according to claim 8.

16. A diagnostic kit for the detection of an infection by a pathogenic strain of *Streptococcus suis*, characterised by containing at least one polypeptide according to claim 9.

17. A diagnostic kit for the detection of an infection by a pathogenic strain of *Streptococcus suis*, characterised by containing at least one antibody according to claim 10.

18. A method for protecting mammals against an infection by *Streptococcus suis*, characterised by using a polynucleotide according to any one of claims 2-7, a polypeptide according to claim 9, or an antibody according to claim 10.

19. A method for protecting mammals against an infection by *Streptococcus suis*, characterised by using a polypeptide according to claim 9 lacking the parts which are responsible for virulence.

20. Vaccine which protects mammals against an infection by *Streptococcus suis*, containing a polynucleotide according to claim 7, a polypeptide according to claim 9, or an antibody according to claim 10.

21. Vaccine which protects mammals against an infection by *Streptococcus suis*, containing a polypeptide used in the method according to claim 19.

22. Vaccine which protects mammals against an infection by *Streptococcus suis*, containing a material of a *Streptococcus suis* strain which material does not express at least a polypeptide according to claim 9.

=o=o=o=

Fig 1a.1

SEQ ID NO: 1
SEQUENCE TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 4376 base pairs
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: genomic DNA
ORIGINAL SOURCE ORGANISM: *Streptococcus suis* type II (pathogenic)
PROPERTIES: Extracellular protein factor (EF) gene

FEATURES:
from bp 66 to 71: promoter -35 region
from bp 89 to 94: promoter -10 region
from bp 153 to 158: promoter -35 region
from bp 176 to 181: promoter -10 region
from bp 350 to 356: ribosome binding site
from bp 361 to 498: signal peptide
from bp 499 to 2890: mature peptide
from bp 4186 to 4198 and from bp 4203 to 4215: dyad symmetry regions
from bp 4243 to 4257 and from bp 4263 to 4276: dyad symmetry regions

```
TTGAACAACT TAAAACTAGT TAGTTTTGTT TAAAATGTAA TTGAATTGTC TTTTTAAGTA   60
GGCTGTTTAC ACGATATTTG TCTTCCTTTA TATAAATATG ATAGATTTTC AGTAAATTTT  120
TCAAAAAAAC CTCAAAAATA ACAGATTTTT TCTTGTATCT TTGAGGCATA AGGAGTATAA  180
TGGTGACGGT ATTCAAGTAG AAATTTTATA TACTCTTGAT GAAAACATTC TGTCTACTTT  240
AAAATAAATA ATCTACTGGG TATCCTTCTG CTAAGTTTTT AAAGCAGGAG GTGTGTTTTT  300
GTACATGGTG TTACAGGAAC CAGAAATGAT CGATTCGCCA GTAAAATATA GGAGGATATC  360

ATG TCT TAT AAA GAT ATG TTC AGA AAA GAA CAA CGT TTT TCT TTT CGT    408
Met Ser Tyr Lys Asp Met Phe Arg Lys Glu Gln Arg Phe Ser Phe Arg
    -45                 -40                 -35

AAA TTT AGC TTT GGT CTA GCT TCG GCA GTC ATT GCA AAC GTT ATT TTG    456
Lys Phe Ser Phe Gly Leu Ala Ser Ala Val Ile Ala Asn Val Ile Leu
-30                 -25                 -20                 -15

GGA GGA GCA ATC GCA AAC AGC CCT GTT GTT CAT GCT AAC ACA GTG ACA    504
Gly Gly Ala Ile Ala Asn Ser Pro Val Val His Ala Asn Thr Val Thr
                -10                  -5                   1

GAA GCA GAG ACA GCT GTA GCA CCA GCT AAC CAA GAC CTT GGA AAT GAG    552
Glu Ala Glu Thr Ala Val Ala Pro Ala Asn Gln Asp Leu Gly Asn Glu
         5                  10                  15

ACT AAA ACG GAA GAA GAA CCC AAG GAA CCA ATC GAA GCA GTT CGC ACG    600
Thr Lys Thr Glu Glu Glu Pro Lys Glu Pro Ile Glu Ala Val Arg Thr
    20                  25                  30
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1a.2

GAC ATG GAA AAC CGT GCA GCT GAA ATC TTG CCG GAG GCG CTG AAT GCT 648
Asp Met Glu Asn Arg Ala Ala Glu Ile Leu Pro Glu Ala Leu Asn Ala
35 40 45 50

AGT GTA ACA AAC CAA GCA CCA GTT ATT CCG ACT ATT GGA GAT CTT CCT 696
Ser Val Thr Asn Gln Ala Pro Val Ile Pro Thr Ile Gly Asp Leu Pro
55 60 65

AAA GAT GCG AGT GGT CAG AAT GTT CAT GGT AAG GCA ACG GAT AAT AAG 744
Lys Asp Ala Ser Gly Gln Asn Val His Gly Lys Ala Thr Asp Asn Lys
70 75 80

ATT TAT CGT GTT GTA TAC GTT TTT GGT AAT GTA GCA GGG ACT ACG GAG 792
Ile Tyr Arg Val Val Tyr Val Phe Gly Asn Val Ala Gly Thr Thr Glu
85 90 95

ACA GAA GAT GGT AAA CAA AAT GTT GCT CCA ACA TTT AAC AGA AAT GAT 840
Thr Glu Asp Gly Lys Gln Asn Val Ala Pro Thr Phe Asn Arg Asn Asp
100 105 110

GCA ACT AAA ACT TTT CCA ATC ACA GAT CCA GAT AGC GAC ATT CAA ACT 888
Ala Thr Lys Thr Phe Pro Ile Thr Asp Pro Asp Ser Asp Ile Gln Thr
115 120 125 130

ATT TCA TAC GAA GTT CCA GCT GAT ATT GCA AGC TAT ACC TTG GAT GAT 936
Ile Ser Tyr Glu Val Pro Ala Asp Ile Ala Ser Tyr Thr Leu Asp Asp
135 140 145

CCA AAC TCA ATT GTT ACT AAT GGC ACC TCA CCT GGT CCA GTA TCT TAC 984
Pro Asn Ser Ile Val Thr Asn Gly Thr Ser Pro Gly Pro Val Ser Tyr
150 155 160

TTA GAT GGT CCA AAT GGG TCA GCC ACT CTC ACA CAA GAT GGT TAT CTA 1032
Leu Asp Gly Pro Asn Gly Ser Ala Thr Leu Thr Gln Asp Gly Tyr Leu
165 170 175

ACA GGA AGT TTC CCT TGG GGA GCA GGA GAC CTA GCT GGT CGT CGG ATT 1080
Thr Gly Ser Phe Pro Trp Gly Ala Gly Asp Leu Ala Gly Arg Arg Ile
180 185 190

AAA GTG ACG GAT GCC ACT GGT AAT ACT ACT AAG AGT AAT CCG TTC TAT 1128
Lys Val Thr Asp Ala Thr Gly Asn Thr Thr Lys Ser Asn Pro Phe Tyr
195 200 205 210

ATG GTT GCA TAT ACA GTC AAG CCA GTA GAT GAT AAA CCT CTA GCA GTA 1176
Met Val Ala Tyr Thr Val Lys Pro Val Asp Asp Lys Pro Leu Ala Val
215 220 225

TCA AAC TCT TCT GAG CTG ACG GAA CAG GCT ATT TTT GAT AAG TTG GTT 1224
Ser Asn Ser Ser Glu Leu Thr Glu Gln Ala Ile Phe Asp Lys Leu Val
230 235 240

1a.3

GTC GAT AAG TCT GCT AAA ACA ACT TCA AAT AGC GCT CTT GTA ATT GAT 1272
Val Asp Lys Ser Ala Lys Thr Thr Ser Asn Ser Ala Leu Val Ile Asp
245 250 255

TCT AGC AAC TAC AAA CAT TCA ATT GCA GGT TAT CGT ACC GTA AAT TCT 1320
Ser Ser Asn Tyr Lys His Ser Ile Ala Gly Tyr Arg Thr Val Asn Ser
260 265 270

GAT GGC ACA AAA ACA GAA ACA GTA GAG GAA ACA AAT CTA TCT GAT TTC 1368
Asp Gly Thr Lys Thr Glu Thr Val Glu Glu Thr Asn Leu Ser Asp Phe
275 280 285 290

CCA ACT GAA GGT AAA TAC GAA GTT CGA GTA AAA ACA ACC AAT GTT TAC 1416
Pro Thr Glu Gly Lys Tyr Glu Val Arg Val Lys Thr Thr Asn Val Tyr
295 300 305

GGT CAA ACT ATC TAC AAC TGG ATT CCT GTA AAT GCC TAT AAG TTG GAC 1464
Gly Gln Thr Ile Tyr Asn Trp Ile Pro Val Asn Ala Tyr Lys Leu Asp
310 315 320

ACA GCG AAG GAT GCT GAA ATT CGG AAG TAT ACA GAC AAC CAA GCC CCA 1512
Thr Ala Lys Asp Ala Glu Ile Arg Lys Tyr Thr Asp Asn Gln Ala Pro
325 330 335

ATT CAT GCT ATA ATG CAA ATT GGT CAA GCT GGA GAA AAG GCA GCA GTT 1560
Ile His Ala Ile Met Gln Ile Gly Gln Ala Gly Glu Lys Ala Ala Val
340 345 350

ATA TTG AAG GAT ATT CCA TCC GAT TTC AGT ATT GAA AAC TTC AAT TTG 1608
Ile Leu Lys Asp Ile Pro Ser Asp Phe Ser Ile Glu Asn Phe Asn Leu
355 360 365 370

AAA GAT GGT GTA GCA GAT GAG CTT GCT AAA CGT AAC TTG GAA TTT GTA 1656
Lys Asp Gly Val Ala Asp Glu Leu Ala Lys Arg Asn Leu Glu Phe Val
375 380 385

AGA AAT GAT GCA GTG GCG ACA ACT GAT ACT GAT GGA GAT GGC GCC AAA 1704
Arg Asn Asp Ala Val Ala Thr Thr Asp Thr Asp Gly Asp Gly Ala Lys
390 395 400

GAA GGA ATT GTT GGA TAT ATT CAA CCA AAA ACT GGC GGT GCA AAC AGT 1752
Glu Gly Ile Val Gly Tyr Ile Gln Pro Lys Thr Gly Gly Ala Asn Ser
405 410 415

GGG GTA GCC ACT TAT ACA GGA TCA AAT AAT CTT ACT TAT GGC TTC ACT 1800
Gly Val Ala Thr Tyr Thr Gly Ser Asn Asn Leu Thr Tyr Gly Phe Thr
420 425 430

TAC AAA GCT GTT GAG ACA AAA GAT AAG GCG AAT GCC ACA GAG GCT AAA 1848
Tyr Lys Ala Val Glu Thr Lys Asp Lys Ala Asn Ala Thr Glu Ala Lys
435 440 445 450

1a.4

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ACT CTC GAA TTA GAT TAC ACC ATC TTA TTC ATA GAT ACT AAA GCA CCA   1896
Thr Leu Glu Leu Asp Tyr Thr Ile Leu Phe Ile Asp Thr Lys Ala Pro
                455                 460                 465

GTC ATG ACA CCT AAA TCA GAG TAC ATC CGT TTT GTT GGT GAA GAG TAT   1944
Val Met Thr Pro Lys Ser Glu Tyr Ile Arg Phe Val Gly Glu Glu Tyr
            470                 475                 480

ACG GTT AGC GTC CCA GGT ACG GAT AAC GCC TTC CTT AAT ACC GGC AAA   1992
Thr Val Ser Val Pro Gly Thr Asp Asn Ala Phe Leu Asn Thr Gly Lys
        485                 490                 495

CTA AAT GGA ACT CTC TCA ATT TTG AAA GAT GGA GAG TCA GGT TCT CTT   2040
Leu Asn Gly Thr Leu Ser Ile Leu Lys Asp Gly Glu Ser Gly Ser Leu
    500                 505                 510

GTA TCA TCA GAC TTA GGT ACA AAC ACT AAG ATT ACT TCA GAA CTG GAT   2088
Val Ser Ser Asp Leu Gly Thr Asn Thr Lys Ile Thr Ser Glu Leu Asp
515                 520                 525                 530

CCT ACG GGA GCA ACT GCA AAC CAA GGA GAT GAC GGT CAA TCT TCA ACT   2136
Pro Thr Gly Ala Thr Ala Asn Gln Gly Asp Asp Gly Gln Ser Ser Thr
                535                 540                 545

AAG TTT AAC GTT AAG ATT ACA GGT ACC GGA CCT GCT ACA GAA GGT ACC   2184
Lys Phe Asn Val Lys Ile Thr Gly Thr Gly Pro Ala Thr Glu Gly Thr
            550                 555                 560

GGC ACT TAT AAG CTT CGT GTT GGA GAA GAT AAC TAT CCT TTT GGT CCA   2232
Gly Thr Tyr Lys Leu Arg Val Gly Glu Asp Asn Tyr Pro Phe Gly Pro
        565                 570                 575

GAG GGG AAA CTT GTT GAT GGA AAT AAA CCA GAA AAT GTA GGT TTG ACA   2280
Glu Gly Lys Leu Val Asp Gly Asn Lys Pro Glu Asn Val Gly Leu Thr
    580                 585                 590

TCT GTA AAA GTT ACC TTC GTA AAA CAT GCT ACG GTG TCA ACA CCA GTT   2328
Ser Val Lys Val Thr Phe Val Lys His Ala Thr Val Ser Thr Pro Val
595                 600                 605                 610

TCT GTT GAA AAT CCA GCT AAC TTA ACG CCA GAA GAA AAA GCC GCA GTT   2376
Ser Val Glu Asn Pro Ala Asn Leu Thr Pro Glu Glu Lys Ala Ala Val
                615                 620                 625

ATT GCT CAA ATC AAG AAA GAC AAC GCA GAC AAC GAA AGA TTG AAG GGC   2424
Ile Ala Gln Ile Lys Lys Asp Asn Ala Asp Asn Glu Arg Leu Lys Gly
            630                 635                 640

TTG CCA GAT TCA GCA TTT ACA GTT AAC TCA GAT GGT ACT GTG TCA GTT   2472
Leu Pro Asp Ser Ala Phe Thr Val Asn Ser Asp Gly Thr Val Ser Val
        645                 650                 655
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1a.5

GAC TAC AGT GCC GGT GGT GTC AAT GTT GAT GGT GCG ACA GAC ATT ATT 2520
Asp Tyr Ser Ala Gly Gly Val Asn Val Asp Gly Ala Thr Asp Ile Ile
660 665 670

AAG AAT GCT ACC ACA AAC TTG GCA GAT ACA CGG AAT GAA GCA AAA GCA 2568
Lys Asn Ala Thr Thr Asn Leu Ala Asp Thr Arg Asn Glu Ala Lys Ala
675 680 685 690

GAA ATC GAC ACA AAA TTA GCT GAA CAT AAA AAA GCT ATC GAA GCA AAA 2616
Glu Ile Asp Thr Lys Leu Ala Glu His Lys Lys Ala Ile Glu Ala Lys
695 700 705

CGG GAT GAA GCG TTT TCT AAA ATT GAT GAT GAC ATT TCC TTG AGA GCA 2664
Arg Asp Glu Ala Phe Ser Lys Ile Asp Asp Asp Ile Ser Leu Arg Ala
710 715 720

GAA CAG AGA CAG GCT GCT AAG GAT GCC GTT GCT GCA GCT GCT GGG GAT 2712
Glu Gln Arg Gln Ala Ala Lys Asp Ala Val Ala Ala Ala Ala Gly Asp
725 730 735

GCT TTG AAA GAA TTA GAC AAC AAG GCG ACA GAA GCA AAA GAA AAA ATT 2760
Ala Leu Lys Glu Leu Asp Asn Lys Ala Thr Glu Ala Lys Glu Lys Ile
740 745 750

GAT AAA GCT ACG ACG GCC TCA GAA ATC AAT GAT GCT AAG ACT AAT GGT 2808
Asp Lys Ala Thr Thr Ala Ser Glu Ile Asn Asp Ala Lys Thr Asn Gly
755 760 765 770

GAG ATT AAT CTG GAC AGT GCA GAA GCA GTA GGC GAA AAA GCT ATT AAC 2856
Glu Ile Asn Leu Asp Ser Ala Glu Ala Val Gly Glu Lys Ala Ile Asn
775 780 785

CAG TCG AAG CGC AAT CGG CAG AGG ACA AAG GCG TAG GT TCA ATC GCC 2903
Gln Ser Lys Arg Asn Arg Gln Arg Thr Lys Ala -
790 795

CA GTC GAA GCG CAA TCG GCA GAG GAC AAA GGC GTA GGT TCA ATC GCC 2903
Val Glu Ala Gln Ser Ala Glu Asp Lys Gly Val Gly Ser Ile Ala

CAA GAT GTT CTT GAC GCA GCG AAA CAA GAT GCT AAG AAT AAG ATT GCT 2951
Gln Asp Val Leu Asp Ala Ala Lys Gln Asp Ala Lys Asn Lys Ile Ala

AAA GAA TCC GAC GCT GCT AAG TCA GCC ATT GAC GCG AAT CCA AAC TTG 2999
Lys Glu Ser Asp Ala Ala Lys Ser Ala Ile Asp Ala Asn Pro Asn Leu

ACA GAT GCA GAG AAG GAA TCA GCT AAG AAA GCG GTA GAT GCA GAT GCT 3047
Thr Asp Ala Glu Lys Glu Ser Ala Lys Lys Ala Val Asp Ala Asp Ala

AAA GCT GCG ACA GAT GCA ATT GAT GCT TCA ACA AGT CCA GTC GAA GCG 3095
Lys Ala Ala Thr Asp Ala Ile Asp Ala Ser Thr Ser Pro Val Glu Ala

1a.6

CAA TCG GCA GAG GAC AAA GGC GTA GGC GCC ATC GCC AAA GAC ATT CTT 3143
Gln Ser Ala Glu Asp Lys Gly Val Gly Ala Ile Ala Lys Asp Ile Leu

GAT GCC GCG AAA CAA GAT GCT AAG AAC AAG ATT GCT AAA GAG GCA GAA 3191
Asp Ala Ala Lys Gln Asp Ala Lys Asn Lys Ile Ala Lys Glu Ala Glu

TCC GCT AAG TCA GTC ATT GAC TCC AAT CCG AAC TTG ACA GAT GCA GCT 3239
Ser Ala Lys Ser Val Ile Asp Ser Asn Pro Asn Leu Thr Asp Ala Ala

AAG GAA GCG GCT AAA TCT GAA ATT GAT AAA GCT GTT GAG GAA GCG ATT 3287
Lys Glu Ala Ala Lys Ser Glu Ile Asp Lys Ala Val Glu Glu Ala Ile

GTT TTA ATC AAT GGT GTT AGA ACT TAT CAA GAG TTG GAA AAA ATC AAA 3335
Val Leu Ile Asn Gly Val Arg Thr Tyr Gln Glu Leu Glu Lys Ile Lys

CTT CCA ATG GCA GCT CTA ATT AAA CCA GCT GCG AAA GTA ACA CCA GTG 3383
Leu Pro Met Ala Ala Leu Ile Lys Pro Ala Ala Lys Val Thr Pro Val

GTT GAT CCA AAT AAC TTG ACT GAA AAA GAA ATT GCT CGT ATC AAG GCA 3431
Val Asp Pro Asn Asn Leu Thr Glu Lys Glu Ile Ala Arg Ile Lys Ala

TTC CTT AAA GAG AAC AAT AAC CTC CCA TAA GGA ACA GAG ATT AAT GTT 3479
Phe Leu Lys Glu Asn Asn Asn Leu Pro - Gly Thr Glu Ile Asn Val

TCT AAA GAT GCT TCA GTG ACA ATT AAA TAT CCA GAT GGA ACT ATT GAT 3527
Ser Lys Asp Ala Ser Val Thr Ile Lys Tyr Pro Asp Gly Thr Ile Asp

TTG CTA TCA CCA GTA GAA GTT GTG AAG CAG GCA GAT AAA ACT GCT CCT 3575
Leu Leu Ser Pro Val Glu Val Val Lys Gln Ala Asp Lys Thr Ala Pro

ACG GTC GCA AAT GAT GGC AAA GGT AAT ATT GTG ATT GTA CCG TCT GAA 3623
Thr Val Ala Asn Asp Gly Lys Gly Asn Ile Val Ile Val Pro Ser Glu

AAA GCT GTT GAG CTT GTT GTT TCA TAC GTA GAT AAC AAT GGT AAG TCG 3671
Lys Ala Val Glu Leu Val Val Ser Tyr Val Asp Asn Asn Gly Lys Ser

CAA ACT GTA GTT GTT ACG AAA GGT ACG GAT GGT TTA TGG ACA GCA AGT 3719
Gln Thr Val Val Val Thr Lys Gly Thr Asp Gly Leu Trp Thr Ala Ser

AAT ACA GTG GTG ATT GTG GAC CCT GTG ACT GGG CAA GTA ATC GTT CCA 3767
Asn Thr Val Val Ile Val Asp Pro Val Thr Gly Gln Val Ile Val Pro

GGT TCT GTT ATT AAG CCA GGT ACA GTT GTT ACA GCA TAC TCT AAA GAC 3815
Gly Ser Val Ile Lys Pro Gly Thr Val Val Thr Ala Tyr Ser Lys Asp

GAG GTT GGA AAT AGT TCT GAT TCA GCA GAA GCT GAA GTT GTA GCA GTA 3863
Glu Val Gly Asn Ser Ser Asp Ser Ala Glu Ala Glu Val Val Ala Val

GAC GAA AAT AAT TCT GCA GCA GGA GTG AAA GTT AAA TCA GTT ACT ACA 3911
Asp Glu Asn Asn Ser Ala Ala Gly Val Lys Val Lys Ser Val Thr Thr

1a.7

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AAT GCT AAT AAT GTT GAG AAG AAA GCT AAG CAA TTA CCG AAT ACT GGT   3959
Asn Ala Asn Asn Val Glu Lys Lys Ala Lys Gln Leu Pro Asn Thr Gly

GAG GAA GCA AAT TCA GCA ACT TCA CTC GGA TTA GTA GCT CTT GGA CTC   4007
Glu Glu Ala Asn Ser Ala Thr Ser Leu Gly Leu Val Ala Leu Gly Leu

GGA TTA GCA CTT CTT GCA GCA AAG AGA AGA AGA GAC GAA GAA GCT TAA   4055
Gly Leu Ala Leu Leu Ala Ala Lys Arg Arg Arg Asp Glu Glu Ala  -

GATAAGCTCT TCCTCAGAAC TCTTTTGGAA GCCGCAATTT TCCTAGAAGA TAGTAGTATG 4115
ATACTCTTTC ATAGCAAGGA AATTCCCTCG CTATGATTGG TAGGTATCAG TTATTATCTA 4175
TCGAACCCCC AAAATCCAAA GTCATTCGAC TTTGGATTTT TTTGATACGA CATGCTCGTC 4235
           >>>>>>>>>> >>>     <<< <<<<<<<<<<
ATACCTAAAA AACAGCCTTC TCTTGCCGAG AGGCTGTTTT TCATGCTTTT AATCTAAAAG 4295
       >>> >>>>>>>>>> >>      <<< <<<<<<<<<< <
TCTGCGGACG TTTTTTCAAT AAAATCCAGT AACCGATGCT AACATAGGCA ATCATAGCTA 4355
GGGAAACCAG CAGGATATAG G                                           4376
```

Fig. 1b. 1

SEQ ID NO: 2
SEQUENCE TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 6744 base pairs
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: genomic DNA
ORIGINAL SOURCE ORGANISM: *Streptococcus suis* type II (non-pathogenic)
PROPERTIES: Extracellular factor related protein (EF*) gene

FEATURES:
from bp 66 to 71: promoter -35 region
from bp 89 to 94: promoter -10 region
from bp 153 to 158: promoter -35 region
from bp 176 to 181: promoter -10 region
from bp 350 to 356: ribosome binding site
from bp 361 to 498: signal peptide
from bp 499 to 5826: mature peptide
bp 2869, 3097, 3292, 3520, 4087, 4381, 4609, 4837, 5065, 5293, 5521: start of repetitive units R1 - R11
bp 2932, 3160, 3355, 3583, 4150, 4444, 4672, 4900, 5128, 5356, 5584: start of repetitive Asn-Pro-Asn-Leu sequences
from bp 6554 to 6566 and from bp 6571 to 6583: dyad symmetry regions
from bp 6611 to 6625 and from bp 6631 to 6644: dyad symmetry regions

```
TTGAACAACT TAAAACTAGT TAGTTTTGTT TAAAATGTAA TTGAATTGTC TTTTTAAGTA  60
GGCTGTTTAC ACGATATTTG TCTTCCTTTA TATAAATATG ATAGATTTTC AGTAAATTTT  120
TCAAAAAAAC CTCAAAAATA ACAGATTTTT TCTTGTATCT TTGAGGCATA AGGAGTATAA  180
TGGTGACGGT ATTCAAGTAG AAATTTTATA TACTCTTGAT GAAAACATTC TGTCTACTTT  240
AAAATAAATA ATCTACTGGG TATCCTTCTG CTAAGTTTTT AAAGCAGGAG GTGTGTTTTT  300
GTACATGGTG TTACAGGAAC CAGAAATGAT CGATTCGCCA GTAAAATATA GGAGGATATC  360

ATG TCT TAT AAA GAT ATG TTC AGA AAA GAA CAA CGT TTT TCT TTT CGT   408
Met Ser Tyr Lys Asp Met Phe Arg Lys Glu Gln Arg Phe Ser Phe Arg
    -45                 -40                 -35

AAA TTT AGC TTT GGT CTA GCT TCG GCA GTC ATT GCA AAC GTT ATT TTG   456
Lys Phe Ser Phe Gly Leu Ala Ser Ala Val Ile Ala Asn Val Ile Leu
-30                 -25                 -20                 -15

GGA GGA GCA ATC GCA AAC AGC CCT GTT GTT CAT GCT AAC ACA GTG ACA   504
Gly Gly Ala Ile Ala Asn Ser Pro Val Val His Ala Asn Thr Val Thr
                -10                 -5                  1

GAA GCA GAG ACA GCT GTA GCA CCA GCT AAC CAA GAC CTT GGA AAT GAG   552
Glu Ala Glu Thr Ala Val Ala Pro Ala Asn Gln Asp Leu Gly Asn Glu
        5                   10                  15
```

1b.2

```
ACT AAA ACG GAA GAA GAA CCC AAG GAA CCA ATC GAA GCA GTT CGC ACG    600
Thr Lys Thr Glu Glu Glu Pro Lys Glu Pro Ile Glu Ala Val Arg Thr
     20                  25                  30

GAC ATG GAA AAC CGT GCA GCT GAA ATC TTG CCG GAG GCG CTG AAT GCT    648
Asp Met Glu Asn Arg Ala Ala Glu Ile Leu Pro Glu Ala Leu Asn Ala
35                  40                  45                  50

AGT GTA ACA AAC CAA GCA CCA GTT ATT CCG ACT ATT GGA GAT CTT CCT    696
Ser Val Thr Asn Gln Ala Pro Val Ile Pro Thr Ile Gly Asp Leu Pro
                55                  60                  65

AAA GAT GCG AGT GGT CAG AAT GTT CAT GGT AAG GCA ACG GAT AAT AAG    744
Lys Asp Ala Ser Gly Gln Asn Val His Gly Lys Ala Thr Asp Asn Lys
            70                  75                  80

ATT TAT CGT GTT GTA TAC GTT TTT GGT AAT GTA GCA GGG ACT ACG GAG    792
Ile Tyr Arg Val Val Tyr Val Phe Gly Asn Val Ala Gly Thr Thr Glu
        85                  90                  95

ACA GAA GAT GGT AAA CAA AAT GTT GCT CCA ACA TTT AAC AGA AAT GAT    840
Thr Glu Asp Gly Lys Gln Asn Val Ala Pro Thr Phe Asn Arg Asn Asp
    100                 105                 110

GCA ACT AAA ACT TTT CCA ATC ACA GAT CCA GAT AGC GAC ATT CAA ACT    888
Ala Thr Lys Thr Phe Pro Ile Thr Asp Pro Asp Ser Asp Ile Gln Thr
115                 120                 125                 130

ATT TCA TAC GAA GTT CCA GCT GAT ATT GCA AGC TAT ACC TTG GAT GAT    936
Ile Ser Tyr Glu Val Pro Ala Asp Ile Ala Ser Tyr Thr Leu Asp Asp
                135                 140                 145

CCA AAC TCA ATT GTT ACT AAT GGC ACC TCA CCT GGT CCA GTA TCT TAC    984
Pro Asn Ser Ile Val Thr Asn Gly Thr Ser Pro Gly Pro Val Ser Tyr
            150                 155                 160

TTA GAT GGT CCA AAT GGG TCA GCC ACT CTC ACA CAA GAT GGT TAT CTA    1032
Leu Asp Gly Pro Asn Gly Ser Ala Thr Leu Thr Gln Asp Gly Tyr Leu
        165                 170                 175

ACA GGA AGT TTC CCT TGG GGA GCA GGA GAC CTA GCT GGT CGT CGG ATT    1080
Thr Gly Ser Phe Pro Trp Gly Ala Gly Asp Leu Ala Gly Arg Arg Ile
    180                 185                 190

AAA GTG ACG GAT GCC ACT GGT AAT ACT ACT AAG AGT AAT CCG TTC TAT    1128
Lys Val Thr Asp Ala Thr Gly Asn Thr Thr Lys Ser Asn Pro Phe Tyr
195                 200                 205                 210

ATG GTT GCA TAT ACA GTC AAG CCA GTA GAT GAT AAA CCT CTA GCA GTA    1176
Met Val Ala Tyr Thr Val Lys Pro Val Asp Asp Lys Pro Leu Ala Val
                215                 220                 225
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1b.3

TCA AAC TCT TCT GAG CTG ACG GAA CAG GCT ATT TTT GAT AAG TTG GTT 1224

Ser Asn Ser Ser Glu Leu Thr Glu Gln Ala Ile Phe Asp Lys Leu Val

230 235 240

GTC GAT AAG TCT GCT AAA ACA ACT TCA AAT AGC GCT CTT GTA ATT GAT 1272

Val Asp Lys Ser Ala Lys Thr Thr Ser Asn Ser Ala Leu Val Ile Asp

245 250 255

TCT AGC AAC TAC AAA CAT TCA ATT GCA GGT TAT CGT ACC GTA AAT TCT 1320

Ser Ser Asn Tyr Lys His Ser Ile Ala Gly Tyr Arg Thr Val Asn Ser

260 265 270

GAT GGC ACA AAA ACA GAA ACA GTA GAG GAA ACA AAT CTA TCT GAT TTC 1368

Asp Gly Thr Lys Thr Glu Thr Val Glu Glu Thr Asn Leu Ser Asp Phe

275 280 285 290

CCA ACT GAA GGT AAA TAC GAA GTT CGA GTA AAA ACA ACC AAT GTT TAC 1416

Pro Thr Glu Gly Lys Tyr Glu Val Arg Val Lys Thr Thr Asn Val Tyr

295 300 305

GGT CAA ACT ATC TAC AAC TGG ATT CCT GTA AAT GCC TAT AAG TTG GAC 1464

Gly Gln Thr Ile Tyr Asn Trp Ile Pro Val Asn Ala Tyr Lys Leu Asp

310 315 320

ACA GCG AAG GAT GCT GAA ATT CGG AAG TAT ACA GAC AAC CAA GCC CCA 1512

Thr Ala Lys Asp Ala Glu Ile Arg Lys Tyr Thr Asp Asn Gln Ala Pro

325 330 335

ATT CAT GCT ATA ATG CAA ATT GGT CAA GCT GGA GAA AAG GCA GCA GTT 1560

Ile His Ala Ile Met Gln Ile Gly Gln Ala Gly Glu Lys Ala Ala Val

340 345 350

ATA TTG AAG GAT ATT CCA TCC GAT TTC AGT ATT GAA AAC TTC AAT TTG 1608

Ile Leu Lys Asp Ile Pro Ser Asp Phe Ser Ile Glu Asn Phe Asn Leu

355 360 365 370

AAA GAT GGT GTA GCA GAT GAG CTT GCT AAA CGT AAC TTG GAA TTT GTA 1656

Lys Asp Gly Val Ala Asp Glu Leu Ala Lys Arg Asn Leu Glu Phe Val

375 380 385

AGA AAT GAT GCA GTG GCG ACA ACT GAT ACT GAT GGA GAT GGC GCC AAA 1704

Arg Asn Asp Ala Val Ala Thr Thr Asp Thr Asp Gly Asp Gly Ala Lys

390 395 400

GAA GGA ATT GTT GGA TAT ATT CAA CCA AAA ACT GGC GGT GCA AAC AGT 1752

Glu Gly Ile Val Gly Tyr Ile Gln Pro Lys Thr Gly Gly Ala Asn Ser

405 410 415

GGG GTA GCC ACT TAT ACA GGA TCA AAT AAT CTT ACT TAT GGC TTC ACT 1800

Gly Val Ala Thr Tyr Thr Gly Ser Asn Asn Leu Thr Tyr Gly Phe Thr

420 425 430

1b. 4

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TAC AAA GCT GTT GAG ACA AAA GAT AAG GCG AAT GCC ACA GAG GCT AAA   1848
Tyr Lys Ala Val Glu Thr Lys Asp Lys Ala Asn Ala Thr Glu Ala Lys
435                 440                 445                 450

ACT CTC GAA TTA GAT TAC ACC ATC TTA TTC ATA GAT ACT AAA GCA CCA   1896
Thr Leu Glu Leu Asp Tyr Thr Ile Leu Phe Ile Asp Thr Lys Ala Pro
                455                 460                 465

GTC ATG ACA CCT AAA TCA GAG TAC ATC CGT TTT GTT GGT GAA GAG TAT   1944
Val Met Thr Pro Lys Ser Glu Tyr Ile Arg Phe Val Gly Glu Glu Tyr
            470                 475                 480

ACG GTT AGC GTC CCA GGT ACG GAT AAC GCC TTC CTT AAT ACC GGC AAA   1992
Thr Val Ser Val Pro Gly Thr Asp Asn Ala Phe Leu Asn Thr Gly Lys
        485                 490                 495

CTA AAT GGA ACT CTC TCA ATT TTG AAA GAT GGA GAG TCA GGT TCT CTT   2040
Leu Asn Gly Thr Leu Ser Ile Leu Lys Asp Gly Glu Ser Gly Ser Leu
    500                 505                 510

GTA TCA TCA GAC TTA GGT ACA AAC ACT AAG ATT ACT TCA GAA CTG GAT   2088
Val Ser Ser Asp Leu Gly Thr Asn Thr Lys Ile Thr Ser Glu Leu Asp
515                 520                 525                 530

CCT ACG GGA GCA ACT GCA AAC CAA GGA GAT GAC GGT CAA TCT TCA ACT   2136
Pro Thr Gly Ala Thr Ala Asn Gln Gly Asp Asp Gly Gln Ser Ser Thr
                535                 540                 545

AAG TTT AAC GTT AAG ATT ACA GGT ACC GGA CCT GCT ACA GAA GGT ACC   2184
Lys Phe Asn Val Lys Ile Thr Gly Thr Gly Pro Ala Thr Glu Gly Thr
            550                 555                 560

GGC ACT TAT AAG CTT CGT GTT GGA GAA GAT AAC TAT CCT TTT GGT CCA   2232
Gly Thr Tyr Lys Leu Arg Val Gly Glu Asp Asn Tyr Pro Phe Gly Pro
        565                 570                 575

GAG GGG AAA CTT GTT GAT GGA AAT AAA CCA GAA AAT GTA GGT TTG ACA   2280
Glu Gly Lys Leu Val Asp Gly Asn Lys Pro Glu Asn Val Gly Leu Thr
    580                 585                 590

TCT GTA AAA GTT ACC TTC GTA AAA CAT GCT ACG GTG TCA ACA CCA GTT   2328
Ser Val Lys Val Thr Phe Val Lys His Ala Thr Val Ser Thr Pro Val
595                 600                 605                 610

TCT GTT GAA AAT CCA GCT AAC TTA ACG CCA GAA GAA AAA GCC GCA GTT   2376
Ser Val Glu Asn Pro Ala Asn Leu Thr Pro Glu Glu Lys Ala Ala Val
                615                 620                 625

ATT GCT CAA ATC AAG AAA GAC AAC GCA GAC AAC GAA AGA TTG AAG GGC   2424
Ile Ala Gln Ile Lys Lys Asp Asn Ala Asp Asn Glu Arg Leu Lys Gly
            630                 635                 640
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1b. 5

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TTG CCA GAT TCA GCA TTT ACA GTT AAC TCA GAT GGT ACT GTG TCA GTT    2472
Leu Pro Asp Ser Ala Phe Thr Val Asn Ser Asp Gly Thr Val Ser Val
        645                 650                 655

GAC TAC AGT GCC GGT GGT GTC AAT GTT GAT GGT GCG ACA GAC ATT ATT    2520
Asp Tyr Ser Ala Gly Gly Val Asn Val Asp Gly Ala Thr Asp Ile Ile
    660                 665                 670

AAG AAT GCT ACC ACA AAC TTG GCA GAT ACA CGG AAT GAA GCA AAA GCA    2568
Lys Asn Ala Thr Thr Asn Leu Ala Asp Thr Arg Asn Glu Ala Lys Ala
675                 680                 685                 690

GAA ATC GAC ACA AAA TTA GCT GAA CAT AAA AAA GCT ATC GAA GCA AAA    2616
Glu Ile Asp Thr Lys Leu Ala Glu His Lys Lys Ala Ile Glu Ala Lys
                695                 700                 705

CGG GAT GAA GCG TTT TCT AAA ATT GAT GAT GAC ATT TCC TTG AGA GCA    2664
Arg Asp Glu Ala Phe Ser Lys Ile Asp Asp Asp Ile Ser Leu Arg Ala
            710                 715                 720

GAA CAG AGA CAG GCT GCT AAG GAT GCC GTT GCT GCA GCT GCT GGG GAT    2712
Glu Gln Arg Gln Ala Ala Lys Asp Ala Val Ala Ala Ala Ala Gly Asp
        725                 730                 735

GCT TTG AAA GAA TTA GAC AAC AAG GCG ACA GAA GCA AAA GAA AAA ATT    2760
Ala Leu Lys Glu Leu Asp Asn Lys Ala Thr Glu Ala Lys Glu Lys Ile
    740                 745                 750

GAT AAA GCT ACG ACG GCC TCA GAA ATC AAT GAT GCT AAG ACT AAT GGT    2808
Asp Lys Ala Thr Thr Ala Ser Glu Ile Asn Asp Ala Lys Thr Asn Gly
755                 760                 765                 770

GAG ATT AAT CTG GAC AGT GCA GAA GCA GTA GGC GAA AAA GCT ATT AAC    2856
Glu Ile Asn Leu Asp Ser Ala Glu Ala Val Gly Glu Lys Ala Ile Asn
                775                 780                 785

CAG GCG AAG GAA AAA GAA CTG GCA AAA GCA GAA GTT GAA AAC AAA GCA    2904
Gln Ala Lys Glu Lys Glu Leu Ala Lys Ala Glu Val Glu Asn Lys Ala
            790                 795                 800

TTC GAG GCA TTG GAA AAA GTT AAC AAT AAT CCA AAC TTG TTA GAA GAA    2952
Phe Glu Ala Leu Glu Lys Val Asn Asn Asn Pro Asn Leu Leu Glu Glu
        805                 810                 815

GAG AAA AAA GCA TAC TTT GAT GAT ATT AAA GAA TCT AAA GAA GTT GCA    3000
Glu Lys Lys Ala Tyr Phe Asp Asp Ile Lys Glu Ser Lys Glu Val Ala
    820                 825                 830

GTT GAG AAA ATC AAT AAT GCT GAA AAT ACT GCT GAA ATT ACG GCA GCA    3048
Val Glu Lys Ile Asn Asn Ala Glu Asn Thr Ala Glu Ile Thr Ala Ala
835                 840                 845                 850
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1b. 6

ATT GAC GAA GCG GAA ATT GCA TAC AAT GAA GAT GTT ATT AAC GCA GCC 3096
Ile Asp Glu Ala Glu Ile Ala Tyr Asn Glu Asp Val Ile Asn Ala Ala
855 860 865

CAA CTT GAT GCT TTG AAT AAG CTT GAA AAA GAT AGC GAA GAA ACT AAG 3144
Gln Leu Asp Ala Leu Asn Lys Leu Glu Lys Asp Ser Glu Glu Thr Lys
870 875 880

GCA GCT ATT GAT GCT AAT CCA AAC TTA ACT CCG GAA GAG AAA GCG AAA 3192
Ala Ala Ile Asp Ala Asn Pro Asn Leu Thr Pro Glu Glu Lys Ala Lys
885 890 895

GCT ATT GCT AAG GTA GAA GAG CTT GTT AAT AAT GCT GAA TCT GAC ATT 3240
Ala Ile Ala Lys Val Glu Glu Leu Val Asn Asn Ala Glu Ser Asp Ile
900 905 910

TTG TCG AAG CCT ACC CCA GAA ACA GTT CAA GCA GTG GAG GAT AAG GCT 3288
Leu Ser Lys Pro Thr Pro Glu Thr Val Gln Ala Val Glu Asp Lys Ala
915 920 925 930

GAC AAA GAT CTT GCC AAA GTA GAA CTT CAA GCA GCA GCA GAC GGT GCG 3336
Asp Lys Asp Leu Ala Lys Val Glu Leu Gln Ala Ala Ala Asp Gly Ala
935 940 945

AAG AAA GGC ATT GAA GCA AAT CCG AAT TTG ACT CCA GAA GAG AAA GAT 3384
Lys Lys Gly Ile Glu Ala Asn Pro Asn Leu Thr Pro Glu Glu Lys Asp
950 955 960

GTA GCT AAG AAG GCA GTA GAA GAC GCG GTT AAG GTG GCG ACA GAC GCT 3432
Val Ala Lys Lys Ala Val Glu Asp Ala Val Lys Val Ala Thr Asp Ala
965 970 975

ATT GAT AAG GCG TCA ACT CCA ACC GAA GTT GAC ACA GCG ACA AGC GAT 3480
Ile Asp Lys Ala Ser Thr Pro Thr Glu Val Asp Thr Ala Thr Ser Asp
980 985 990

GGA GTG AAG GCT ATT GAT GCA GAA GAG TTT AAA GCT ACT CAG AAA GAT 3528
Gly Val Lys Ala Ile Asp Ala Glu Glu Phe Lys Ala Thr Gln Lys Asp
995 1000 1005 1010

GCT AAG AAC AAG ATT GCC AAA GAA GCA GAA TCA GCT AAG AAA GCG ATT 3576
Ala Lys Asn Lys Ile Ala Lys Glu Ala Glu Ser Ala Lys Lys Ala Ile
1015 1020 1025

GAC GAC AAT CCA AAC TTG ACT CCA GAT GAG AAG GAA TCA GCT AAG AAT 3624
Asp Asp Asn Pro Asn Leu Thr Pro Asp Glu Lys Glu Ser Ala Lys Asn
1030 1035 1040

GCA GTG GAA GAG GCG GCT AAG GTA GCA ACA GCC GCT ATT GAT AAA GCA 3672
Ala Ser Glu Glu Ala Ala Lys Val Ala Thr Ala Ala Ile Asp Lys Ala
1045 1050 1055

1b.7

TCA ACT CCA GAT GCA GTT CAA GTA GAA GAG GAC AAA GGT GTA GCA GCT 3720
Ser Thr Pro Asp Ala Val Gln Val Glu Glu Asp Lys Gly Val Ala Ala
1060 1065 1070

ATC AAT TTG ATT ACT GCC AAG GCA GAT GCT AAA GGT GTC ATT GCT GCT 3768
Ile Asn Leu Ile Thr Ala Lys Ala Asp Ala Lys Gly Val Ile Ala Ala
1075 1080 1085 1090

AAG TTG GCA GAT GAA ATC AAG AAG CTC GAA GAT AAG CAA GCA GAA GCA 3816
Lys Leu Ala Asp Glu Ile Lys Lys Leu Glu Asp Lys Gln Ala Glu Ala
1095 1100 1105

GAA AAA GCT ATC GAT GCG TCA ACT ATG ACT AAT GAG GAG AAA GCA ATC 3864
Glu Lys Ala Ile Asp Ala Ser Thr Met Thr Asn Glu Glu Lys Ala Ile
1110 1115 1120

GCT AAG AAG GCT CTT CAA GAT GTT GTA GAT AAA GGA AAA GCA GAG CTT 3912
Ala Lys Lys Ala Leu Gln Asp Val Val Asp Lys Gly Lys Ala Glu Leu
1125 1135 1135

GAA GAC GCA GCT AGG GTA GCA ACA AAT GAG ATT CAT GAA GCT ACT ACT 3960
Glu Asp Ala Ala Arg Val Ala Thr Asn Glu Ile His Glu Ala Thr Thr
1140 1145 1150

ACA GAA AAA GCG AAA GCG GCG GAA CTT GCT GGC GAA AAG AGC TTG ACA 4008
Thr Glu Lys Ala Lys Ala Ala Glu Leu Ala Gly Glu Lys Ser Leu Thr
1155 1165 1165 1170

GAC ACA GGT AAA GAA GCT AGA GAT GCA GTT GAA TTG GCT AAG GAT AAA 4056
Asp Thr Gly Lys Glu Ala Arg Asp Ala Val Glu Leu Ala Lys Asp Lys
1175 1180 1185

GAA TTA GCT AAG GAA GCA ATC CGA ACA GAA GAA GAA GAA GCT ACT AAA 4104
Glu Leu Ala Lys Glu Ala Ile Arg Thr Glu Glu Glu Glu Ala Thr Lys
1190 1195 1200

ATA GTA GAG AAA CTT GCA GAA GAT ACG CGC AAA GCT ATC GAG GAC AAT 4152
Ile Val Glu Lys Leu Ala Glu Asp Thr Arg Lys Ala Ile Glu Asp Asn
1205 1210 1215

CCA AAC TTG TCA GAT GAA GAT AAG CAA GCG GAA ATT AAA AAG CTA ACT 4200
Pro Asn Leu Ser Asp Glu Asp Lys Gln Ala Glu Ile Lys Lys Leu Thr
1220 1225 1230

GAC GCT GTG GCA AAA ACT TTA GCA ACC ATT CGT GAC AAT GCA GAT AAG 4248
Asp Ala Val Ala Lys Thr Leu Ala Thr Ile Arg Asp Asn Ala Asp Lys
1235 1240 1245 1250

CGT ACG CAA GAA GCA GAA AAA GCT CAA GCC CTA GCA GAT CTT GAA AAA 4296
Arg Thr Gln Glu Ala Glu Lys Ala Gln Ala Leu Ala Asp Leu Glu Lys
1255 1260 1265

1b.8

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GCT AAA GAA ACA CAG AAA ATT GCA GAT AAA GCT GCG ATT GAT AGG TTG    4344
Ala Lys Glu Thr Gln Lys Ile Ala Asp Lys Ala Ala Ile Asp Arg Leu
            1270                1275                1280

ACT ATA CTT GTG AAA GAT GGT GAG CTT GAA GCT ACT AAA CAA GAT GCT    4392
Thr Ile Leu Val Lys Asp Gly Glu Leu Glu Ala Thr Lys Gln Asp Ala
        1285                1290                1295

AAG AAC AAG ATT GCT AAA GAT GCA GCC GCT GCT AAA GAA GCA ATT GCA    4440
Lys Asn Lys Ile Ala Lys Asp Ala Ala Ala Ala Lys Glu Ala Ile Ala
    1300                1305                1310

AGC AAT CCA AAC TTG ACA GAC GCA GAG AAG AAA ACC TTC ACC GAT GCG    4488
Ser Asn Pro Asn Leu Thr Asp Ala Glu Lys Lys Thr Phe Thr Asp Ala
1315                1320                1325                1330

GTA GAT GCA GAA GTA GCC AAA GCT AAC GAC GCA ATT TCA GCT GCA ACC    4536
Val Asp Ala Glu Val Ala Lys Ala Asn Asp Ala Ile Ser Ala Ala Thr
                1335                1340                1345

AGC CCA GCA GAT GTT CAA AAA GAA GAG GAT GCA GGT GTT GCA GCC ATT    4584
Ser Pro Ala Asp Val Gln Lys Glu Glu Asp Ala Gly Val Ala Ala Ile
            1350                1355                1360

GCA GAA GAT GTT CTT GAC GCA GCT AAA CAA GAT GCT AAG AAT AAG ATT    4632
Ala Glu Asp Val Leu Asp Ala Ala Lys Gln Asp Ala Lys Asn Lys Ile
        1365                1370                1375

GCT AAA GAT GCA GCC GCT GCT AAA GAA GCA ATT GGC TCC AAT CCA AAC    4680
Ala Lys Asp Ala Ala Ala Ala Lys Glu Ala Ile Gly Ser Asn Pro Asn
    1380                1385                1390

TTG ACA GAC GCA GAG AAG AAA ACC TTC ACC GAT GCG GTA GAT GCA GAA    4728
Leu Thr Asp Ala Glu Lys Lys Thr Phe Thr Asp Ala Val Asp Ala Glu
1395                1400                1405                1410

GTA GCC AAA GCT AAC GAC GCA ATT TCA GCT GCA ACC AGC CCA GCA GAT    4776
Val Ala Lys Ala Asn Asp Ala Ile Ser Ala Ala Thr Ser Pro Ala Asp
                1415                1420                1425

GTT CAA AAA GAA GAG GAT GCA GGT GTT GCA GCC ATT GCA GAA GAT GTT    4824
Val Gln Lys Glu Glu Asp Ala Gly Val Ala Ala Ile Ala Glu Asp Val
            1430                1435                1440

CTT GAC GCA GCT AAA CAA GAT GCT AAG AAT AAG ATT GCT AAA GAA TCC    4872
Leu Asp Ala Ala Lys Gln Asp Ala Lys Asn Lys Ile Ala Lys Glu Ser
        1445                1450                1455

GAC GCT GCT AAG TCA GCC ATT GAC GCG AAT CCA AAC TTG ACA GAT GCA    4920
Asp Ala Ala Lys Ser Ala Ile Asp Ala Asn Pro Asn Leu Thr Asp Ala
    1460                1465                1470
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1b.9

GAG AAG GAA TCA GCT AAG AAA GCA GTT GAT GCT GAT GCT AAA GCT GCG 4968
Glu Lys Glu Ser Ala Lys Lys Ala Val Asp Ala Asp Ala Lys Ala Ala
1475 1480 1485 1490

ACA GAT GCA ATT GAT GCT TCA ACA AGT CCA GTC GAA GCG CAA TCG GCA 5016
Thr Asp Ala Ile Asp Ala Ser Thr Ser Pro Val Glu Ala Gln Ser Ala
1495 1500 1505

GAG GAC AAA GGC GTA GGT TCA ATC GCC CAA GAT GTT CTT GAC GCA GCG 5064
Glu Asp Lys Gly Val Gly Ser Ile Ala Gln Asp Val Leu Asp Ala Ala
1510 1515 1520

AAA CAA GAT GCT AAG AAC AAG ATT GCC AAA GAA GTT GCC GCA GCT AAA 5112
Lys Gln Asp Ala Lys Asn Lys Ile Ala Lys Glu Val Ala Ala Ala Lys
1525 1530 1535

GAA GCA ATT GAT GCC AAT CCG AAC TTA TCA GAT GCA GAG AAG GAA GCT 5160
Glu Ala Ile Asp Ala Asn Pro Asn Leu Ser Asp Ala Glu Lys Glu Ala
1540 1545 1550

TCT AAG AAA GCG GTA GAT GCA GAT GCT AAA GCT ACG ACA GAT GCA ATT 5208
Ser Lys Lys Ala Val Asp Ala Asp Ala Lys Ala Thr Thr Asp Ala Ile
1555 1560 1565 1570

GAT GCT TCA ACA AGT CCA GTC GAA GCG CAA TCG GCA GAG GAC AAA GGC 5256
Asp Ala Ser Thr Ser Pro Val Glu Ala Gln Ser Ala Glu Asp Lys Gly
1575 1580 1585

GTA GGT TCA ATC GCC CAA GAT GTT CTT GAC GCA GCG AAA CAA GAT GCT 5304
Val Gly Ser Ile Arg Gln Asp Val Leu Asp Ala Ala Lys Gln Asp Ala
1590 1595 1600

AAG AAT AAG ATT GCT AAA GAA TCC GAC GCT GCT AAG TCA GCC ATT GAC 5352
Lys Asn Lys Ile Ala Lys Glu Ser Asp Ala Ala Lys Ser Ala Ile Asp
1605 1610 1615

GCG AAT CCA AAC TTG ACA GAT GCA GAG AAG GAA TCA GCT AAG AAA GCG 5400
Ala Asn Pro Asn Leu Thr Asp Ala Glu Lys Glu Ser Ala Lys Lys Ala
1620 1625 1630

GTA GAT GCA GAT GCT AAA GCT GCG ACA GAT GCA ATT GAT GCT TCA ACA 5448
Val Asp Ala Asp Ala Lys Ala Ala Thr Asp Ala Ile Asp Ala Ser Thr
1635 1640 1645 1650

AGT CCA GTC GAA GCG CAA TCG GCA GAG GAC AAA GGC GTA GGC GCC ATC 5496
Ser Pro Val Glu Ala Gln Ser Ala Glu Asp Lys Gly Val Gly Ala Ile
1655 1660 1665

GCC AAA GAC ATT CTT GAT GCC GCG AAA CAA GAT GCT AAG AAC AAG ATT 5544
Ala Lys Asp Ile Leu Asp Ala Ala Lys Gln Asp Ala Lys Asn Lys Ile
1670 1675 1680

1b.10

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GCT AAA GAG GCA GAA TCC GCT AAG TCA GTC ATT GAC TCC AAT CCG AAC   5592
Ala Lys Glu Ala Glu Ser Ala Lys Ser Val Ile Asp Ser Asn Pro Asn
        1685                1690                1695

TTG ACA GAT GCA GCT AAG GAA GCG GCT AAA TCT GAA ATT GAT AAA GCT   5640
Leu Thr Asp Ala Ala Lys Glu Ala Ala Lys Ser Glu Ile Asp Lys Ala
    1700                1705                1710

GTT GAG GAA GCG ATT GTT TTA ATC AAT GGT GTT AGA ACT TAT CAA GAG   5688
Val Glu Glu Ala Ile Val Leu Ile Asn Gly Val Arg Thr Tyr Gln Glu
1715                1720                1725                1730

TTG GAA AAA ATC AAA CTT CCA ATG GCA GCT CTA ATT AAA CCA GCT GCG   5736
Leu Glu Lys Ile Lys Leu Pro Met Ala Ala Leu Ile Lys Pro Ala Ala
            1735                1740                1745

AAA GTA ACA CCA GTG GTT GAT CCA AAT AAC TTG ACT GAA AAA GAA ATT   5784
Lys Val Thr Pro Val Val Asp Pro Asn Asn Leu Thr Glu Lys Glu Ile
            1750                1755                1760

GCT CGT ATC AAG GCA TTC CTT AAA GAG AAC AAT AAC CTC CCA TAA       5829
Ala Arg Ile Lys Ala Phe Leu Lys Glu Asn Asn Asn Leu Pro  -
        1765                1770                1775

GGAACAGAGA TTAATGTTTC TAAAGATGCT TCAGTGACAA TTAAATATCC AGATGGAACT 5889
ATTGATTTGC TATCACCAGT AGAAGTTGTG AAGCAGGCAG ATAAAACTGC TCCTACGGTC 5949
GCAAATGATG GCAAAGGTAA TATTGTGATT GTACCGTCTG AAAAAGCTGT TGAGCTTGTT 6009
GTTTCATACG TAGATAACAA TGGTAAGTCG CAAACTGTAG TTGTTACGAA AGGTACGGAT 6069
GGTTTATGGA CAGCAAGTAA TACAGTGGTG ATTGTGGACC CTGTGACTGG GCAAGTAATC 6129
GTTCCAGGTT CTGTTATTAA GCCAGGTACA GTTGTTACAG CATACTCTAA AGACGAGGTT 6189
GGAAATAGTT CTGATTCAGC AGAAGCTGAA GTTGTAGCAG TAGACGAAAA TAATTCTGCA 6249
GCAGGAGTGA AAGTTAAATC AGTTACTACA AATGCTAATA ATGTTGAGAA GAAAGCTAAG 6309
CAATTACCGA ATACTGGTGA GGAAGCAAAT TCAGCAACTT CACTCGGATT AGTAGCTCTT 6369
GGACTCGGAT TAGCACTTCT TGCAGCAAAG AGAAGAAGAG ACGAAGAAGC TTAAGATAAG 6429
CTCTTCCTCA GAACTCTTTT GGAAGCCGCA ATTTTCCTAG AAGATAGTAG TATGATACTC 6489
TTTCATAGCA AGGAAATTCC CTCGCTATGA TTGGTAGGTA TCAGTTATTA TCTATCGAAC 6549
CCCCAAAATC CAAAGTCATT CGACTTTGGA TTTTTTTGAT ACGACATGCT CGTCATACCT 6609
AAAAAAACAGC CTTCTCTTGC CGAGAGGCTG TTTTTCATGC TTTTAATCTA AAAGTCTGCG 6669
GACGTTTTTT CAATAAAATC CAGTAACCGA TGCTAACATA GGCAATCATA GCTAGGGAAA 6729
CCAGCAGGAT ATAGG                                                  6744
```

Fig.2.1

SEQ ID NO: 3
SEQUENCE TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 4118 base pairs
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: genomic DNA
ORIGINAL SOURCE ORGANISM: *Streptococcus suis* type II (pathogenic)
PROPERTIES: Muramidase released protein (MRP) gene

FEATURES:
from bp 4 to 9: promoter -35 region
from bp 29 to 34: promoter -10 region
from bp 40 to 45: promoter -35 region
from bp 63 to 68: promoter -10 region
from bp 147 to 152: ribosome binding site
from bp 159 to 299: signal peptide
from bp 300 to 3926: mature peptide
from bp 2757 to 3014: proline-rich region
from bp 3015 to 3176, 3423 to 3584 and 3585 to 3743: repetitive units
from bp 3825 to 3926: membrane anchor sequence
from bp 4069 to 4080 and from bp 4087 to 4098: dyad symmetry regions

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GAATTCATAA TGTTTTTTTG AGGAATTTTA TAATATTACT TGGCATTTAA AGTTATTTGT    60
AGTATAATAC CTCGAATGAT TGCGGGAGTT TTCAAGGCTT TGATACAAAG AGTAGAAAAT   120
TTGTGTAATT AAATTAATAT TTATATGGGG GATTTTTT                           158

ATG CGT AGA TCA AAT AAA AAA TCA TTT GAC TGG TAC GGT ACG AAA CAA     206
Met Arg Arg Ser Asn Lys Lys Ser Phe Asp Trp Tyr Gly Thr Lys Gln
        -45                 -40                 -35

CAA TTT TCG ATT CGT AAG TAT CAT TTT GGG GCA GCA AGC GTT TTG CTT     254
Gln Phe Ser Ile Arg Lys Tyr His Phe Gly Ala Ala Ser Val Leu Leu
    -30                 -25                 -20

GGT GTG TCG TTA GTT TTA GGT GCT GGT GCA CAG GTT GTT AAG GCT GAT     302
Gly Val Ser Leu Val Leu Gly Ala Gly Ala Gln Val Val Lys Ala Asp
-15                 -10                  -5                   1

GAA ACT GTT GCT TCA TCA GAA CCA ACT ATT GCC AGT AGT GTA GCG CCT     350
Glu Thr Val Ala Ser Ser Glu Pro Thr Ile Ala Ser Ser Val Ala Pro
                5                  10                  15

GCT TCA ACA GAA GCG GTT GCA GAA GAA GCA GAA AAA ACA AAT GCT GAA     398
Ala Ser Thr Glu Ala Val Ala Glu Glu Ala Glu Lys Thr Asn Ala Glu
        20                  25                  30
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2.2

AAT ACG AGT GCA GTA GCT ACG ACT TCA ACA GAA GTT GAA AAA GCG AAA 446
Asn Thr Ser Ala Val Ala Thr Thr Ser Thr Glu Val Glu Lys Ala Lys
35 40 45

GCT GTT CTT GAA CAG GTA ACA TCA GAA TCA CCA CTT TTG GCT GGT CTT 494
Ala Val Leu Glu Gln Val Thr Ser Glu Ser Pro Leu Leu Ala Gly Leu
50 55 60 65

GGT CAA AAA GAG TTG GCT AAA ACT GAA GAT GCA ACT CTT GCA AAA GCT 542
Gly Gln Lys Glu Leu Ala Lys Thr Glu Asp Ala Thr Leu Ala Lys Ala
70 75 80

ATA GAG GAT GCT CAA ACA AAA CTT GCA GCA GCT AAG GCA ATT TTG GCT 590
Ile Glu Asp Ala Gln Thr Lys Leu Ala Ala Ala Lys Ala Ile Leu Ala
85 90 95

GAC TCA GAA GCA ACT GTT GAG CAA GTT GAA GCG CAA GTC GCA GCG GTT 638
Asp Ser Glu Ala Thr Val Glu Gln Val Glu Ala Gln Val Ala Ala Val
100 105 110

AAA GTA GCC AAC GAG GCG CTA GGG AAT GAA TTG CAA AAA TAC ACT GTA 686
Lys Val Ala Asn Glu Ala Leu Gly Asn Glu Leu Gln Lys Tyr Thr Val
115 120 125

GAT GGT CTC TTG ACA GCG GCT CTT GAT ACA GTA GCA CCT GAT ACA ACT 734
Asp Gly Leu Leu Thr Ala Ala Leu Asp Thr Val Ala Pro Asp Thr Thr
130 135 140 145

GCA TCA ACA TTG AAA GTT GGT GAT GGC GAA GGT ACC CTT CTA GAT AGC 782
Ala Ser Thr Leu Lys Val Gly Asp Gly Glu Gly Thr Leu Leu Asp Ser
150 155 160

ACT ACA ACA GCA ACG CCT TCA ATG GCT GAG CCA AAT GGT GCA GCA ATT 830
Thr Thr Thr Ala Thr Pro Ser Met Ala Glu Pro Asn Gly Ala Ala Ile
165 170 175

GCT CCA CAT ACA CTT CGA ACT CAA GAT GGA ATT AAA GCG ACA TCA GAG 878
Ala Pro His Thr Leu Arg Thr Gln Asp Gly Ile Lys Ala Thr Ser Glu
180 185 190

CCA AAT TGG TAT ACT TTT GAA TCG TAC GAT TTG TAC TCA TAT AAT AAA 926
Pro Asn Trp Tyr Thr Phe Glu Ser Tyr Asp Leu Tyr Ser Tyr Asn Lys
195 200 205

AAT ATG GCT AGC TCA ACT TAT AAA GGA GCT GAA GTT GAT GCC TAC ATT 974
Asn Met Ala Ser Ser Thr Tyr Lys Gly Ala Glu Val Asp Ala Tyr Ile
210 215 220 225

CGT TAC TCT TTG GAT AAT GAT TCG TCA ACA ACT GCT GTT TTA GCA GAG 1022
Arg Tyr Ser Leu Asp Asn Asp Ser Ser Thr Thr Ala Val Leu Ala Glu
230 235 240

2.3

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TTG GTA AGT AGG ACA ACT GGT GAT GTG TTA GAG AAA TAT ACG ATT GAA      1070
Leu Val Ser Arg Thr Thr Gly Asp Val Leu Glu Lys Tyr Thr Ile Glu
            245                 250                 255

CCG GGC GAG AGT GTT ACG TTT TCA CAT CCG ACA AAA GTT AAT GCT AAT      1118
Pro Gly Glu Ser Val Thr Phe Ser His Pro Thr Lys Val Asn Ala Asn
        260                 265                 270

AAT AGC AAT ATA ACT GTG ACT TAT GAT ACC TCA TTA GCT TCT GCT AAT      1166
Asn Ser Asn Ile Thr Val Thr Tyr Asp Thr Ser Leu Ala Ser Ala Asn
    275                 280                 285

ACT CCT GGA GCA TTG AAA TTC TCT GCT AAT GAT GAT GTT TAT TCA ACA      1214
Thr Pro Gly Ala Leu Lys Phe Ser Ala Asn Asp Asp Val Tyr Ser Thr
290                 295                 300                 305

ATT ATT GTA CCT GCT TAT CAG ATT AAT ACA ACT CGT TAC GTC ACT GAA      1262
Ile Ile Val Pro Ala Tyr Gln Ile Asn Thr Thr Arg Tyr Val Thr Glu
                310                 315                 320

AGT GGC AAA GTT TTG GCA ACC TAT GGT CTT CAA ACT ATT GCA GGA CAG      1310
Ser Gly Lys Val Leu Ala Thr Tyr Gly Leu Gln Thr Ile Ala Gly Gln
            325                 330                 335

GTA GTT ACT CCA TCT TCT GTT CGT GTA TTT ACT GGG TAT GAT TAT GTG      1358
Val Val Thr Pro Ser Ser Val Arg Val Phe Thr Gly Tyr Asp Tyr Val
        340                 345                 350

GCA ACT ACA ACT AAA GCC GTT CAA GGT CCA TAT CCA AAG GGA ACG GTA      1406
Ala Thr Thr Thr Lys Ala Val Gln Gly Pro Tyr Pro Lys Gly Thr Val
    355                 360                 365

TAC CTT GCT GGT ACG GTT CAA AAG GAT ACA GTA CAA TAT AAA GTT ATT      1454
Tyr Leu Ala Gly Thr Val Gln Lys Asp Thr Val Gln Tyr Lys Val Ile
370                 375                 380                 385

CGT GAA ATT GTG GAG AAC GAC CAA GCA GTT CTT AAA TTC TAT TAT TTA      1502
Arg Glu Ile Val Glu Asn Asp Gln Ala Val Leu Lys Phe Tyr Tyr Leu
                390                 395                 400

GAT CCT ACC TAT AAG GGT GAA GTA GAT TGG AGA GGA ACT GAT ACG ACT      1550
Asp Pro Thr Tyr Lys Gly Glu Val Asp Trp Arg Gly Thr Asp Thr Thr
            405                 410                 415

GGG TTT ATT GAG TTG CTT ACA ACT TCC CCA ACA ACC TAT AAA GTT GGT      1598
Gly Phe Ile Glu Leu Leu Thr Thr Ser Pro Thr Thr Tyr Lys Val Gly
        420                 425                 430

ACT ATA TAC GAT TAC AAT ATT AAT TCA AAA ATT ACA GCT CCA TTT ACT      1646
Thr Ile Tyr Asp Tyr Asn Ile Asn Ser Lys Ile Thr Ala Pro Phe Thr
    435                 440                 445
```

2.4

```
ATT GAT CCT ACC AAG AAT GTT ATG GTT TTC AAG GAA AGT GAA CAG AAC    1694
Ile Asp Pro Thr Lys Asn Val Met Val Phe Lys Glu Ser Glu Gln Asn
450                 455                 460                 465

GAG CAA GGT AGC AAA TAT CGC GTC ATT GCT CAA TGG TCA GGA GAT GAA    1742
Glu Gln Gly Ser Lys Tyr Arg Val Ile Ala Gln Trp Ser Gly Asp Glu
                470                 475                 480

ACC ACT AAA GGT ATA TAT GGA AAA ATC TAT ATC GCT ACT CAG GTT TGG    1790
Thr Thr Lys Gly Ile Tyr Gly Lys Ile Tyr Ile Ala Thr Gln Val Trp
            485                 490                 495

ACG ACT AAA TTG GGA ACA AAC GAG TGG GGA TGG TTT GAC TAT TCT GAT    1838
Thr Thr Lys Leu Gly Thr Asn Glu Trp Gly Trp Phe Asp Tyr Ser Asp
        500                 505                 510

GAC CAA GCT GGT ATA AAA TTT AAT AAC AAA GGT TTT TGG CCG GCA GGT    1886
Asp Gln Ala Gly Ile Lys Phe Asn Asn Lys Gly Phe Trp Pro Ala Gly
    515                 520                 525

GTT CAA AAT ACA CTT CGA AAT GCT ACT CCA GCT ACA GCT GTA GAG ACT    1934
Val Gln Asn Thr Leu Arg Asn Ala Thr Pro Ala Thr Ala Val Glu Thr
530                 535                 540                 545

ACT TAT ATC TAC AAA GAA AGT TCC AAG TAT GGT GAT GTC ATT GTT GAG    1982
Thr Tyr Ile Tyr Lys Glu Ser Ser Lys Tyr Gly Asp Val Ile Val Glu
                550                 555                 560

TAC TAC GAT ACT GAC GGA AAA CAA ATT GTA AAT TCA GTT GTA GAT ACT    2030
Tyr Tyr Asp Thr Asp Gly Lys Gln Ile Val Asn Ser Val Val Asp Thr
            565                 570                 575

CCT AAG TCA GCT CTT GGC ACA GAG TAT AAT ACA GAT GTG GAC CGT AGA    2078
Pro Lys Ser Ala Leu Gly Thr Glu Tyr Asn Thr Asp Val Asp Arg Arg
        580                 585                 590

CCA GCC AGC TTG GTT GCT GCT GAT GGG ACA GTC TAC TTC TAC AAA GAA    2126
Pro Ala Ser Leu Val Ala Ala Asp Gly Thr Val Tyr Phe Tyr Lys Glu
    595                 600                 605

GTT AAG TCT GAT TCA GCT AAG ACA ACC GGT ACA GTA GTT GCA GGT ACG    2174
Val Lys Ser Asp Ser Ala Lys Thr Thr Gly Thr Val Val Ala Gly Thr
610                 615                 620                 625

ACA ACT GTT AAG TAT GTT TAC GAA AAA GCT GGT AGC GTT AAT GTT AAC    2222
Thr Thr Val Lys Tyr Val Tyr Glu Lys Ala Gly Ser Val Asn Val Asn
                630                 635                 640

TTC GTT GAC ATC AAT GGT AAA GTA ATC AAA GCT CCT GTT TCA GAT GAA    2270
Phe Val Asp Ile Asn Gly Lys Val Ile Lys Ala Pro Val Ser Asp Glu
            645                 650                 655
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2.5

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Lys Asp Ala Lys Pro Gly Tyr Asn Tyr Asp Thr Asp Leu Asp Gln Lys
660 665 670

TTA GCT TCC ATC ACT TTT GAA GGC AAG GAA TAC AAA CTT GTT CCT GCT 2366
Leu Ala Ser Ile Thr Phe Glu Gly Lys Glu Tyr Lys Leu Val Pro Ala
675 680 685

GGT GAT TAT CCG GTT GGT AAA GTT GGC AAG GGA AAT AAC TTG ATT GAA 2414
Gly Asp Tyr Pro Val Gly Lys Val Gly Lys Gly Asn Asn Leu Ile Glu
690 695 700 705

GTT GGT AAT AAT ACT GCG AAA GGT ATT GAC CCA ACA ACA GGC AAA ATT 2462
Val Gly Asn Asn Thr Ala Lys Gly Ile Asp Pro Thr Thr Gly Lys Ile
710 715 720

GAA GCC GGT GTT AAC AAA GAA GTT ACC TAT GTC TAT AGA GCA GTG ACA 2510
Glu Ala Gly Val Asn Lys Glu Val Thr Tyr Val Tyr Arg Ala Val Thr
725 730 735

GGT TCT GTA GTT GTA AAT TAC AAA GAT ACA GAA GGT AAT GTG ATT AAA 2558
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GAT CCA GAA ACG GAT GTG TCT GAT GCA CCG GTT GGA GAT GCT TAT ACT 2606
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755 760 765

ACA ACT GAC AAG AAA CCA AAC GAA ATC ATC ACA AAA GAT GGA TCA CGC 2654
Thr Thr Asp Lys Lys Pro Asn Glu Ile Ile Thr Lys Asp Gly Ser Arg
770 775 780 785

TAT GTT CTT GTT CCA TCT AAG ACA GAT GGT GAG GAA AAT GGT AAA GTT 2702
Tyr Val Leu Val Pro Ser Lys Thr Asp Gly Glu Glu Asn Gly Lys Val
790 795 800

ATC GAA GGA ACA ATC ACA GTA ACT TAT GTT TAC CAG AAA GTT GCA AAC 2750
Ile Glu Gly Thr Ile Thr Val Thr Tyr Val Tyr Gln Lys Val Ala Asn
805 810 815

TGG ATT CCA GAG ATT CCA AAT GTA CCA GAA ACA GAC CGT CCA AAA GTA 2798
Trp Ile Pro Glu Ile Pro Asn Val Pro Glu Thr Asp Arg Pro Lys Val
820 825 830

CCT TAC CCA TTT GAC CCA ACA GAG CCA GAC GAG CCA ATC GAT CCA ACG 2846
Pro Tyr Pro Phe Asp Pro Thr Glu Pro Asp Glu Pro Ile Asp Pro Thr
835 840 845

ACA CCA GGA ACA AAT GGC GAG GTT CCA AAT ATT CCT TAC GTT CCA GGA 2894
Thr Pro Gly Thr Asn Gly Glu Val Pro Asn Ile Pro Tyr Val Pro Gly
850 855 860 865

2.6

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Tyr Thr Pro Val Asp Pro Lys Asp Asn Thr Pro Leu Lys Pro Ile Asp
                870                 875                 880

CCA AAT GAT CCA GGT AAG GGT TAT GTA CCA CCA ACA CCA GAA AAT CCA     2990
Pro Asn Asp Pro Gly Lys Gly Tyr Val Pro Pro Thr Pro Glu Asn Pro
            885                 890                 895

GGT GTT GAT ACA CCA ATT CCT TAT GTT CCA GTT AAA AAA GTC GTA ACT     3038
Gly Val Asp Thr Pro Ile Pro Tyr Val Pro Val Lys Lys Val Val Thr
        900                 905                 910

AAC CAC GTT GAT GAA GAG GGT AAC CCT ATT GCA CCG CAA GAA GAG GGA     3086
Asn His Val Asp Glu Glu Gly Asn Pro Ile Ala Pro Gln Glu Glu Gly
    915                 920                 925

ACA AAA CCA AAC AAA TCA ATC CCA GGT TAC GAG TTC ACA GGT AAA ACT     3134
Thr Lys Pro Asn Lys Ser Ile Pro Gly Tyr Glu Phe Thr Gly Lys Thr
930                 935                 940                 945

GTT ACT GAC GAA GAT GGC AAC ACA ACT CAC ATC TAC AAG AAA ACA CCA     3182
Val Thr Asp Glu Asp Gly Asn Thr Thr His Ile Tyr Lys Lys Thr Pro
                950                 955                 960

GAA GTT AAG AAT GGT ACA GTT GTT GTT AAC TAT GTA ACA GAA GAT GGC     3230
Glu Val Lys Asn Gly Thr Val Val Val Asn Tyr Val Thr Glu Asp Gly
            965                 970                 975

ACA GTT ATC AAG GAA CCT GTA ACA GAT ACA CCA ACT TCT CCA GAA GGC     3278
Thr Val Ile Lys Glu Pro Val Thr Asp Thr Pro Thr Ser Pro Glu Gly
        980                 985                 990

ACA CCA TAC GAC ACT ACA GAC AAC AAA CCT AAG ACA ATC ACT TTC AAA     3326
Thr Pro Tyr Asp Thr Thr Asp Asn Lys Pro Lys Thr Ile Thr Phe Lys
    995                 1000                1005

GGT GAA GAG TAT GAA TTG GTT CGT GTT GAC GGT ACA GAA AAC GGT AAA     3374
Gly Glu Glu Tyr Glu Leu Val Arg Val Asp Gly Thr Glu Asn Gly Lys
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GTT GTA GAA GGT GAA ACA GTT GTG ACT TAC GTT TAC CGT AAA GTC GAA     3422
Val Val Glu Gly Glu Thr Val Val Thr Tyr Val Tyr Arg Lys Val Glu
                1030                1035                1040

ACA CCT GCT AAG AAA GTT GTA ACT AAC CAC GTT GAT GAA GAG GGT AAC     3470
Thr Pro Ala Lys Lys Val Val Thr Asn His Val Asp Glu Glu Gly Asn
            1045                1050                1055

CCT GTT GCG CCG CAA GAA GAG GGA ACA AAA CCA AAC AAA TCA ATC CCA     3518
Pro Val Ala Pro Gln Glu Glu Gly Thr Lys Pro Asn Lys Ser Ile Pro
        1060                1065                1070
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2.7

GGT TAC GAA TTT ACA GGT AAA ACT GTT ACT GAC GAA GAT GGC AAC ACA 3566
Gly Tyr Glu Phe Thr Gly Lys Thr Val Thr Asp Glu Asp Gly Asn Thr
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ACT CAC ATC TAC AAG AAA ACA CCT GCT AAG AAA GTT GTG ACT AAC CAC 3614
Thr His Ile Tyr Lys Lys Thr Pro Ala Lys Lys Val Val Thr Asn His
1090 1095 1100 1105

GTT GAT GAA GAA GGT AAC CCT ATT GCT CCA CAA GAG GAT GGG ACA ACA 3662
Val Asp Glu Glu Gly Asn Pro Ile Ala Pro Gln Glu Asp Gly Thr Thr
1110 1115 1120

CCA AAA CGT CAA ATT TCA GGT TAC GAG TAT GTG CGT ACT GTA GTT GAT 3710
Pro Lys Arg Gln Ile Ser Gly Tyr Glu Tyr Val Arg Thr Val Val Asp
1125 1130 1135

GAA GAA GGT AAC ACG ACA CAT ATT TAT CGC AAA CTT TCT AAT AAA CCA 3758
Glu Glu Gly Asn Thr Thr His Ile Tyr Arg Lys Leu Ser Asn Lys Pro
1140 1145 1150

ACA ACA CCT GAG AAG GAA ACT CCT GCA AAA CCT CAA GCA GGT AAA ACC 3806
Thr Thr Pro Glu Lys Glu Thr Pro Ala Lys Pro Gln Ala Gly Lys Thr
1155 1160 1165

GCT TCA GGT AAA GCT CAA TTG CCA AAT ACT GGT GAG GCT TCA TCT GTG 3854
Ala Ser Gly Lys Ala Gln Leu Pro Asn Thr Gly Glu Ala Ser Ser Val
1170 1175 1180 1185

GCA GGT GCG CTT GGT ACA GCA ATG CTT GTC GCA ACA CTT GCG TTT GCA 3902
Ala Gly Ala Leu Gly Thr Ala Met Leu Val Ala Thr Leu Ala Phe Ala
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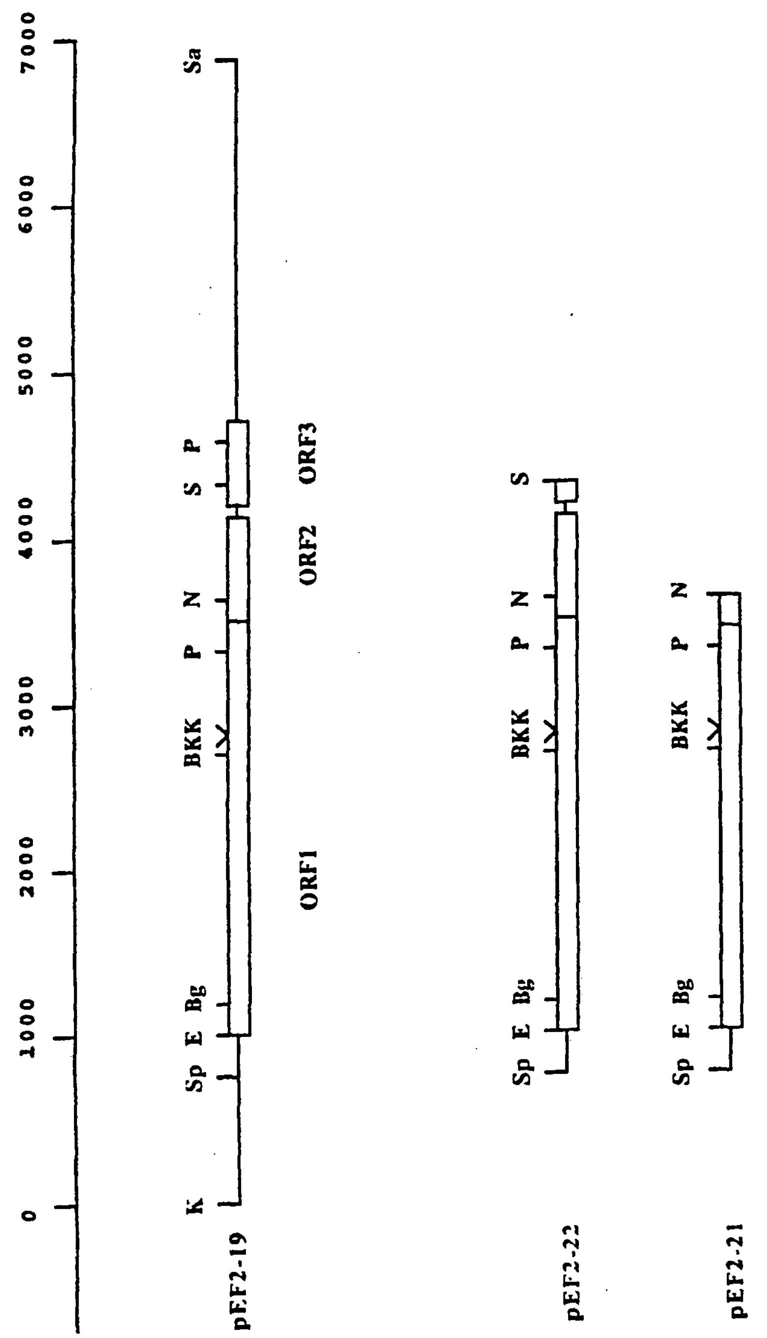
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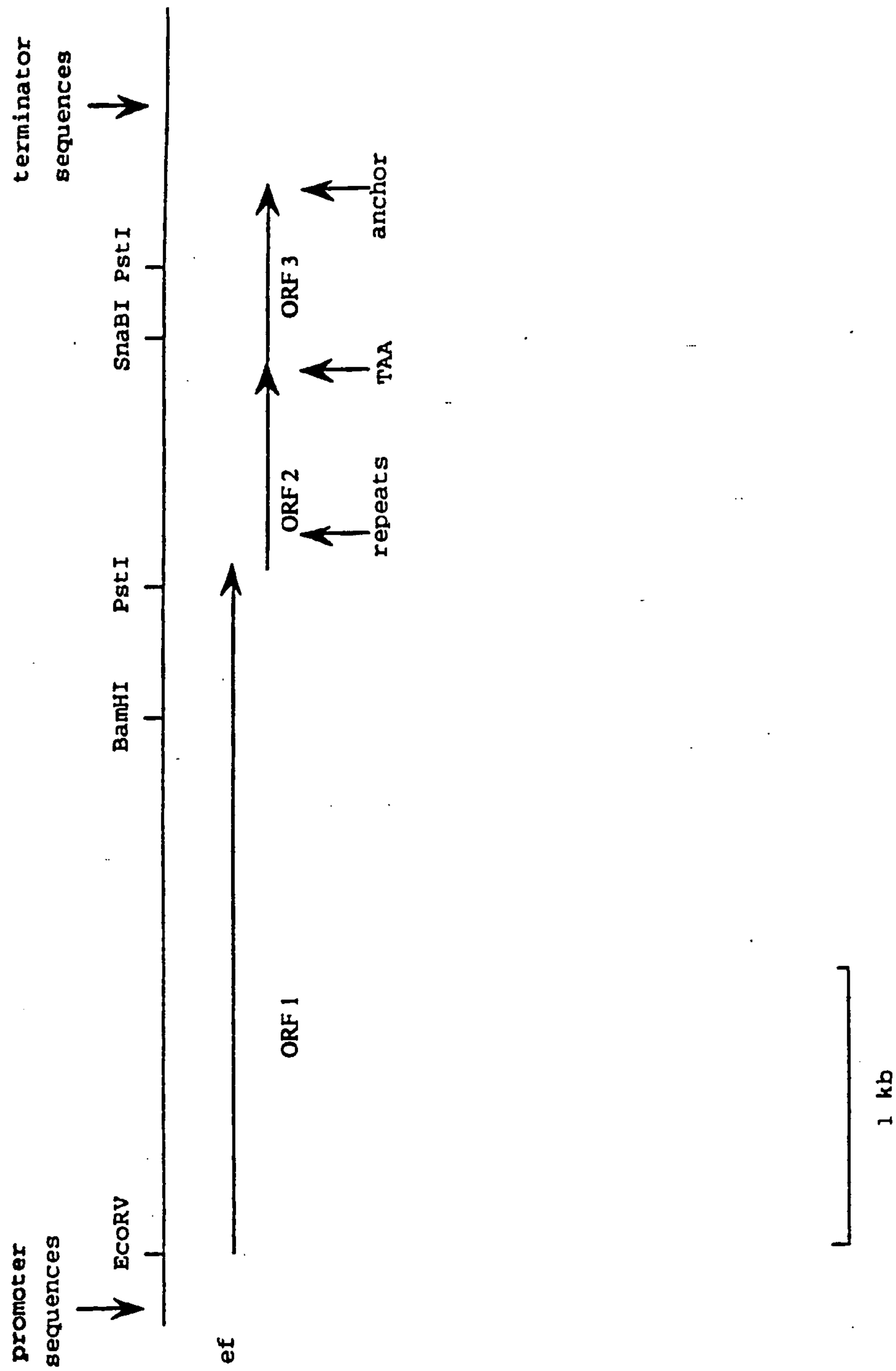
Fig. 3

Fig. 4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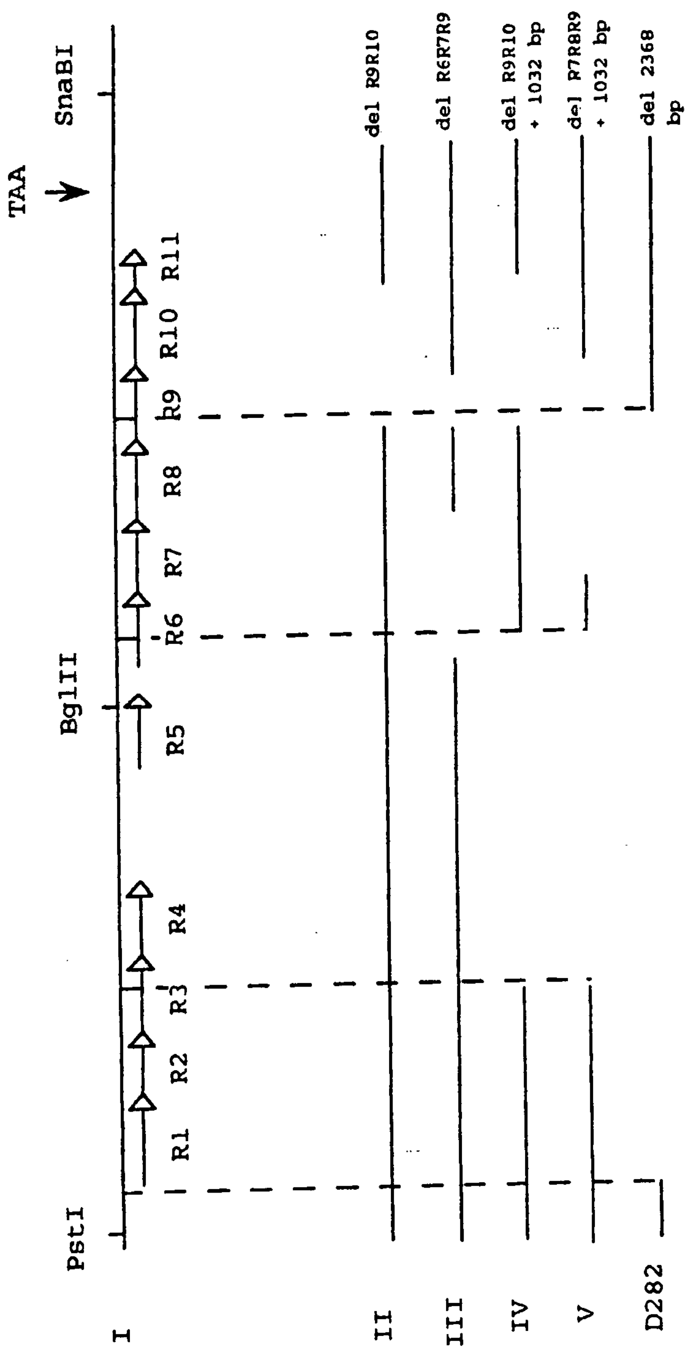
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R11
del R9R10
del R6R7R9
del R9R10
+ 1032 bp
del R7R8R9
+ 1032 bp
del 2368
bp
I
II
III
IV
V
D282

Fig.5

Fig.6

A

3415 -	A A G G T G G C G A C A G A C G C T A T T G A T	left
4447 -	C C A A A C T T G A C A G A C G C A G A G A A G	right
-	A A G G T G G C G A C A G A C G C A G A G A A G	junction

B

2848 -	G C T A T T A A C C A G G C G A A G G A A A A A	left
5216 -	C A A C A A G T C C A G T C G A A G C G C A A T	right
-	G C T A T T A A C C A G T C G A A G C G C A A T	junction

Fig.7

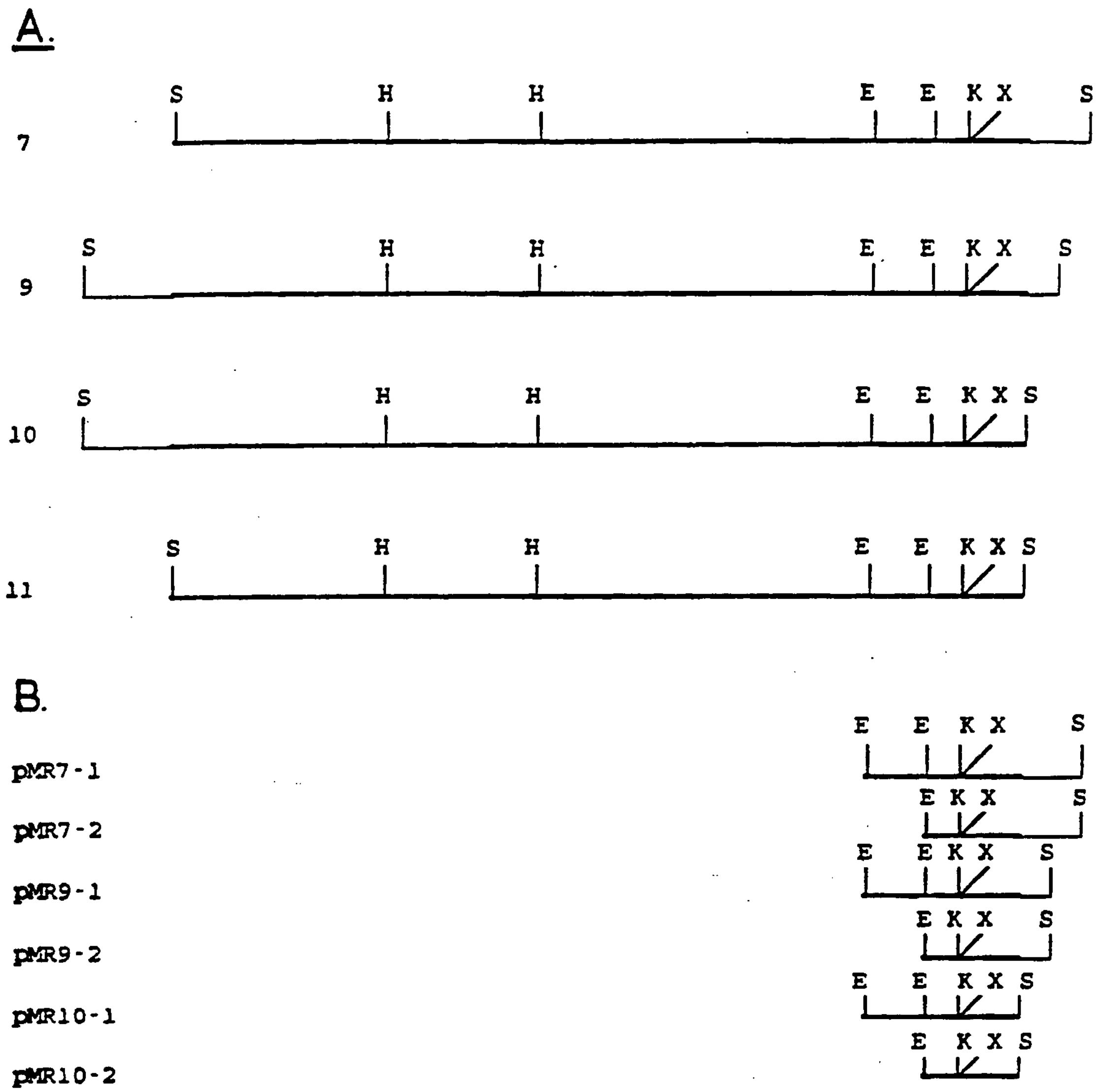

A.
S H H E E K X S
7
S H H E E K X S
9
S H H E E K X S
10
S H H E E K X S
11
B.
E E K X S
pMR7-1
E K X S
pMR7-2
E E K X S
pMR9-1
E K X S
pMR9-2
E E K X S
pMR10-1
E K X S
pMR10-2

Fig.8

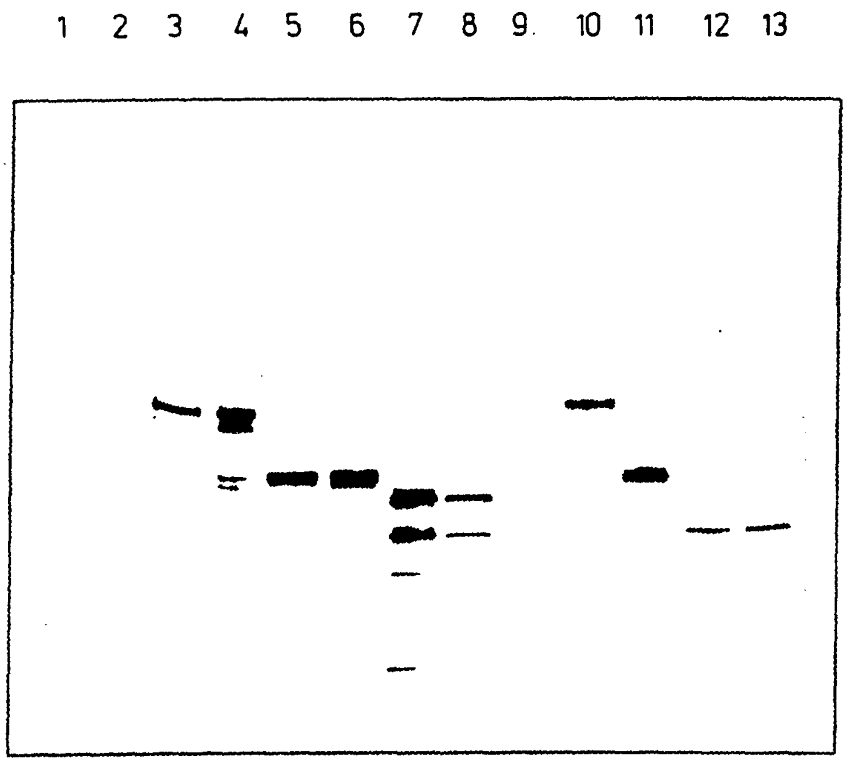
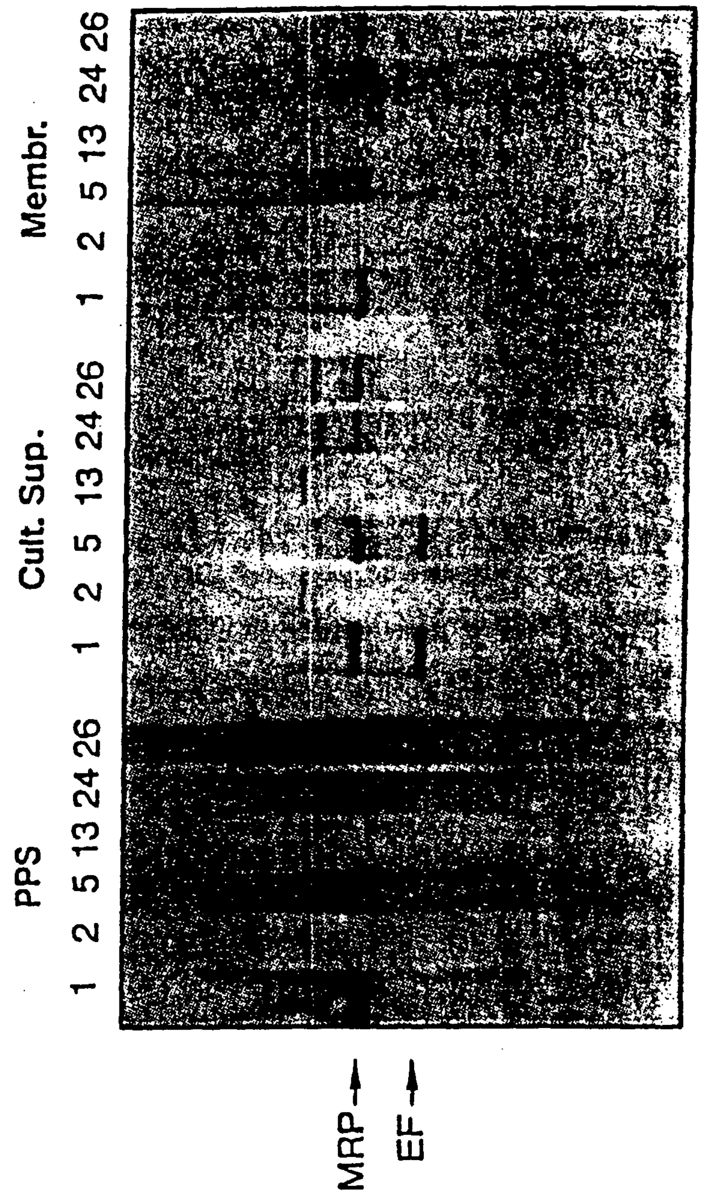


Fig. 9



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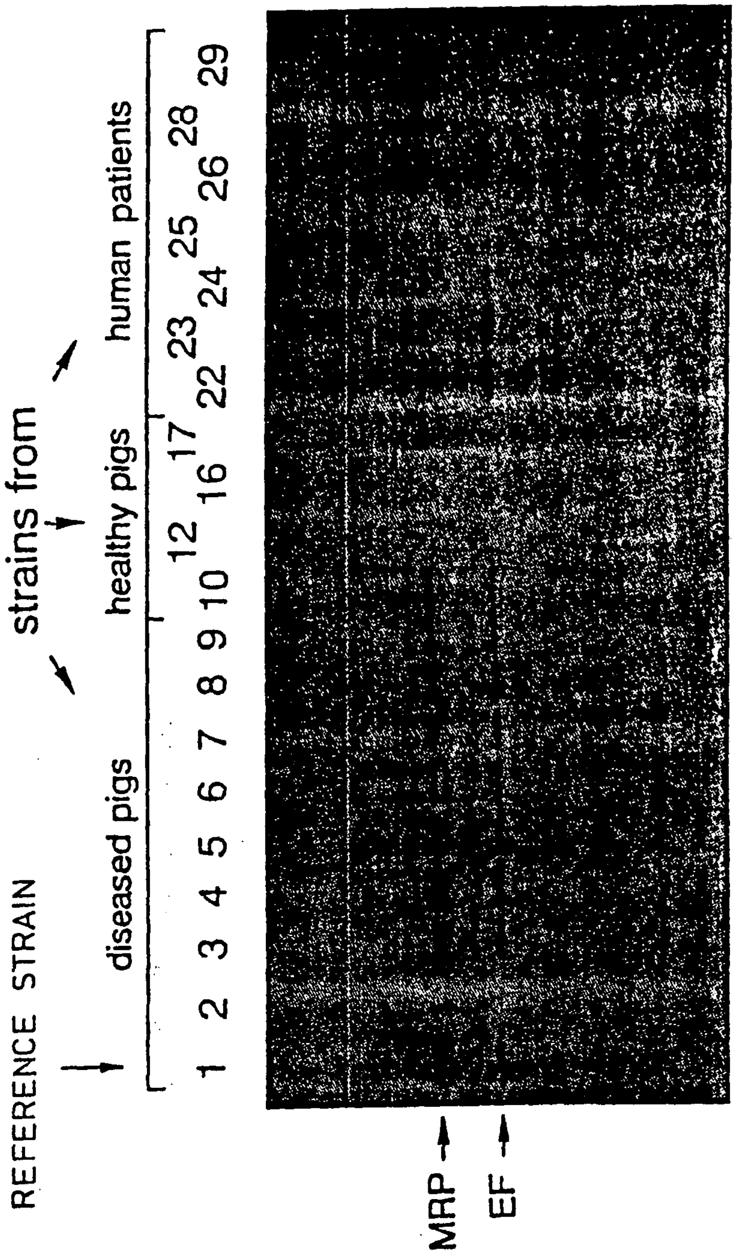
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diseased pigs
healthy pigs
human patients
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MRP
EF

Fig. 11

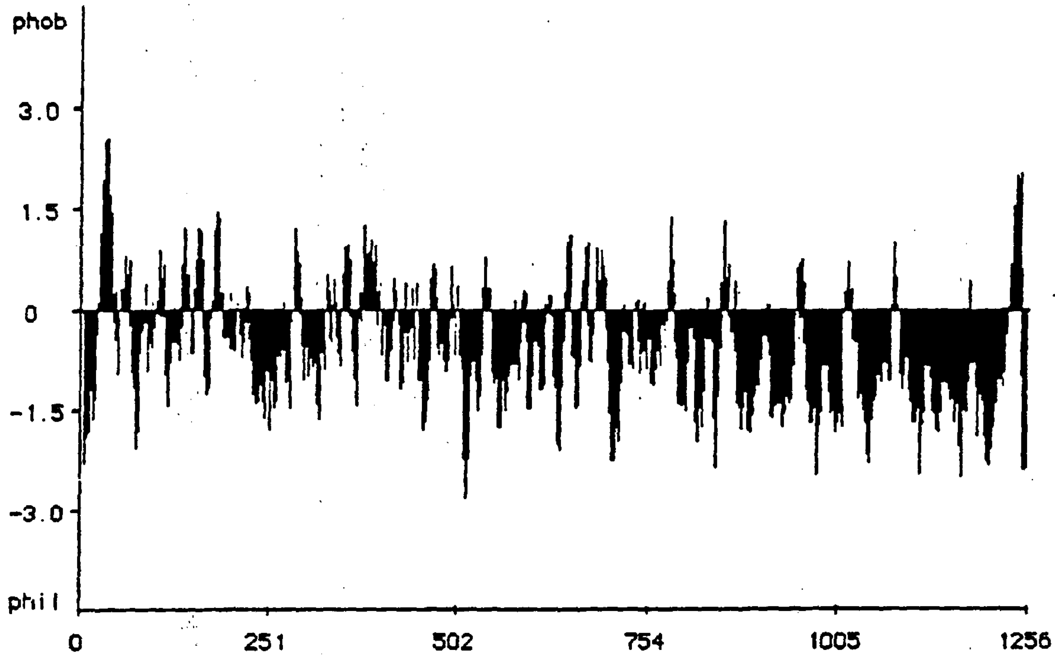

Fig.12

MRP	LP	N	TG	E	----	-	ASSVAGALGTAML	V	ATLAFA	RKRRR	NED*
M6	LP	S	TG	E	TA-N	P	FFTAAALTVMATA	G	VAAVV-	KRK--	EEN*
A	LP	E	TG	E	--EN	P	LIGTTVFGGLSLA	G	AALLAG	RRR--	EL*
G	LP	S	TG	E	GS-N	P	FFTAAALAVMAGA	G	ALAVAS	KRK--	ED*
AP4	LP	S	TG	E	TA-N	P	FFTAAAATVMVSA	G	MLAL--	KRK--	EEN*
LP	LP	K	TG	E	TTEA	P	AFGFLGVIVVSLM	G	VLGV--	KRK--	QAEE*
WAP4	LP	S	TG	E	-QAG	L	LLTTVGLVIVAVA	G	VYFY--	RTRR-	----*
T6	LP	S	TG	S	IGTY	L	FKAIGSAAMIGAI	G	IYIV--	RRK--	A*
Fn-BP	LP	E	TG	G	-EES	T	NKGMLFGGLFSIL	G	LALL--	RRNKK	NHKA*

Fig.13

```
V P V KKVVTNHVDEEGNP I APQE E GT K P NKS I P GYE FT G K TV T DE D GNTTHIYKK
T P A KKVVTNHVDEEGNP V APQE E GT K P NKS I P GYE FT G K TV T DE D GNTTHIYKK
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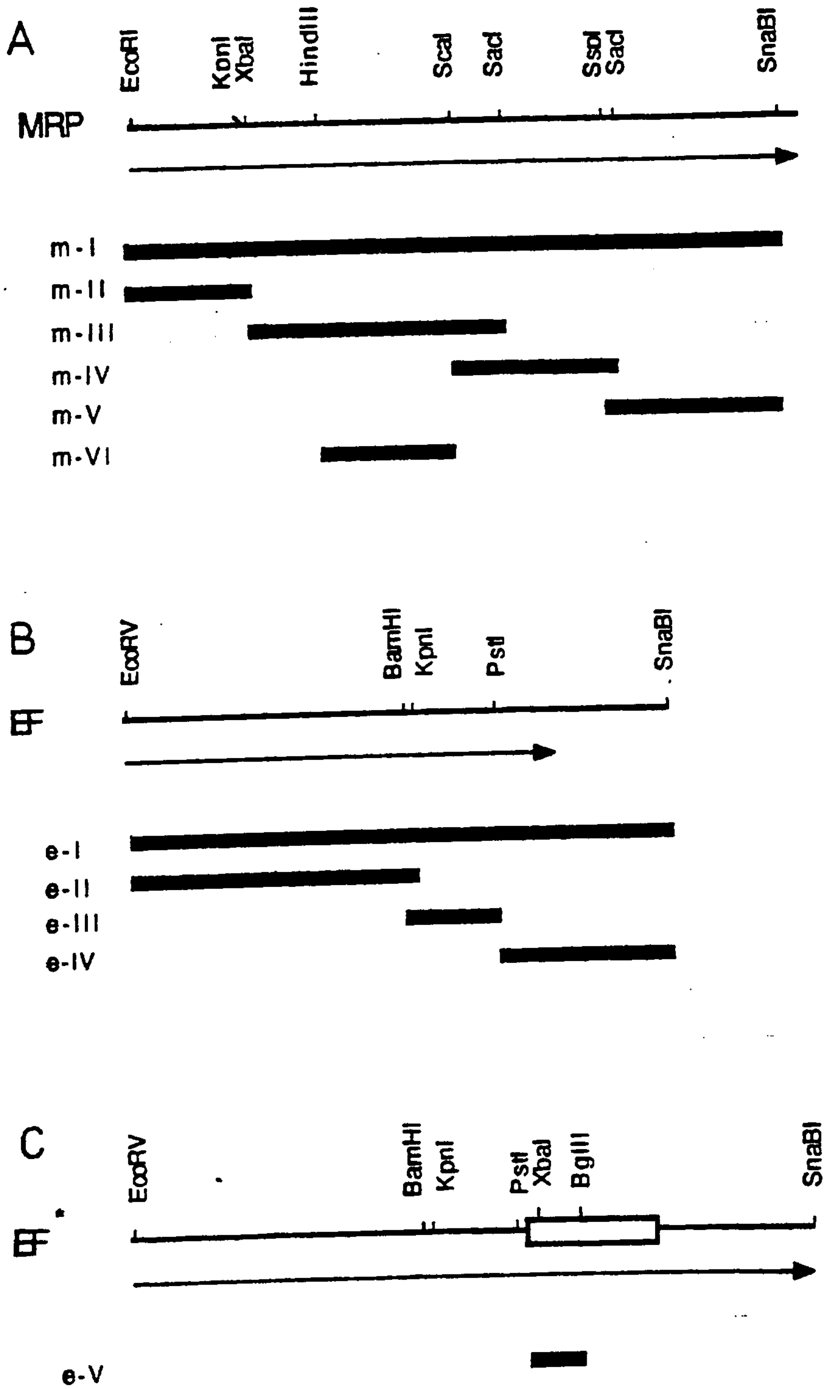
Fig.14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SspI
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m-IV
m-V
m-VI
B
EcoRV
BamHI
KpnI
PstI
SnaBI
EF
e-I
e-II
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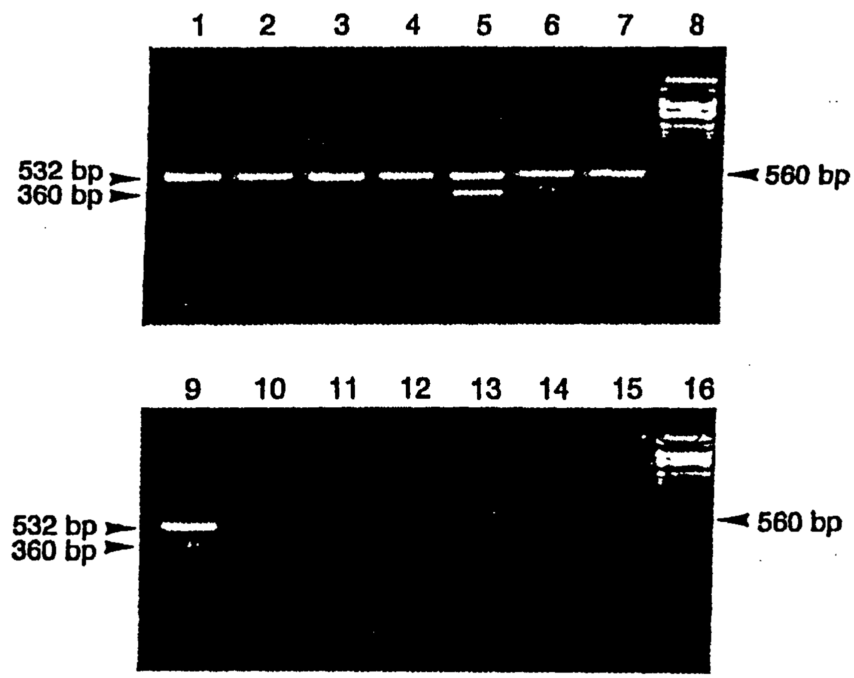
EF*
e-V

Fig. 15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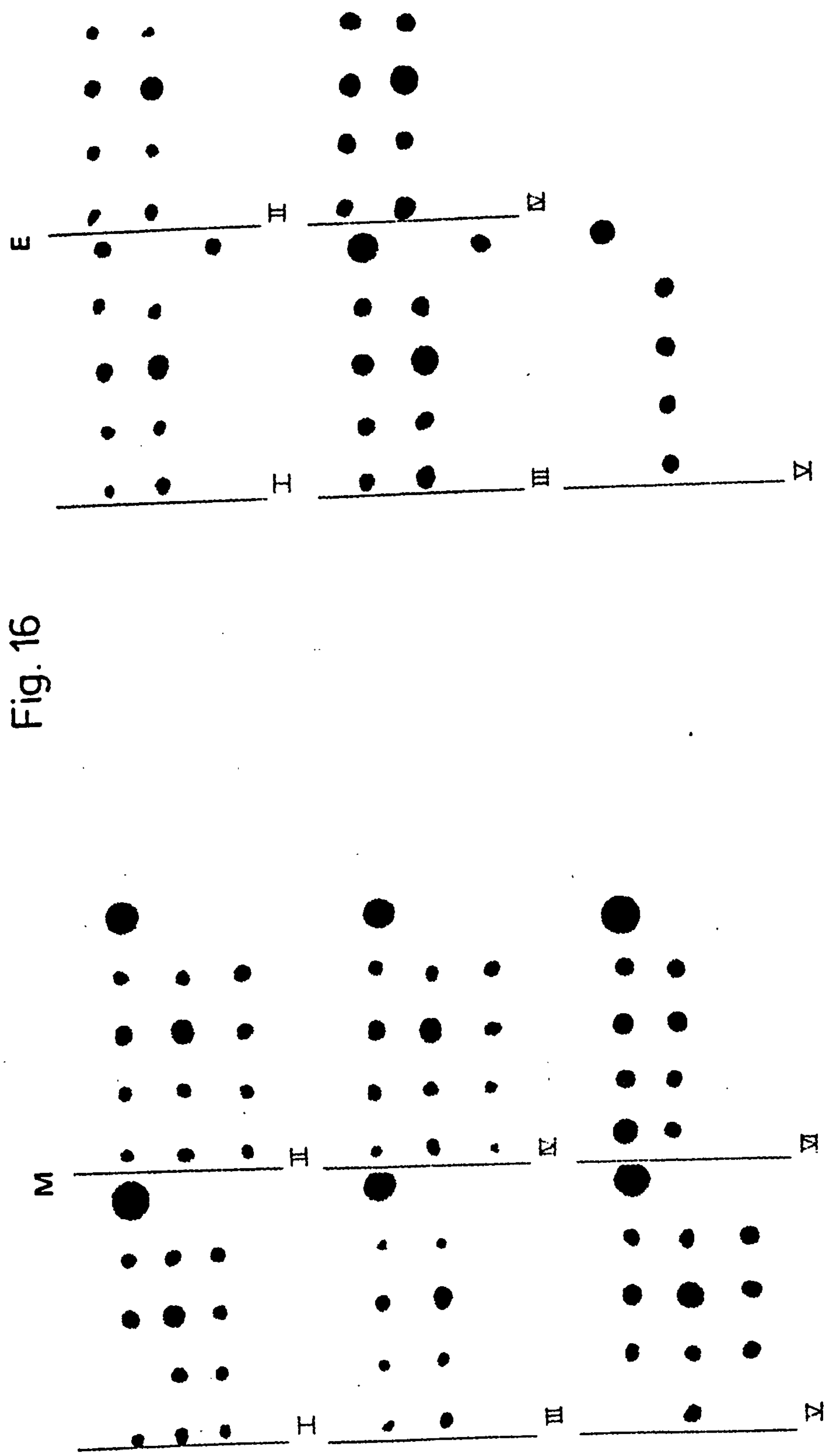

Fig. 16

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 92/00054

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/31 C 07 K 13/00 C 07 K 15/00
C 12 Q 1/68 C 12 Q 1/14 A 61 K 39/09 A 61 K 39/40 //
C 12 N 15/31, C 12 R 1:46

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N C 07 K C 12 Q A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the *Fields Searched*[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	Am. J. Vet. Res., vol. 50, no. 7, July 1989, U. VECHT et al.: "Differences in virulence between two strains of streptococcus suis type II after experimentally induced infection of newborn germ-free pigs", pages 1037-1043, see abstract (cited in the application) ---	1,3,6-22
Y	WO,A,8500832 (THE ROCKEFELLER UNIVERSITY) 28 February 1985, see the whole document ---	1,3,6-22
Y	Abstracts of Papers, part 1, 200th ACS National Meeting, Washington, DC, 26-31 August 1990, American Chemical Society, J.R. LOWE et al.: "PCR-detection of virulent Bacillus anthracis strains", abstract no. 158, see abstract --- -/-	1,3,6-9,11-17

° *Special categories of cited documents* : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17-06-1992	16. 07. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	N. TORIBIO NURIA TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0383509 (ORTHO DIAGNOSTIC SYSTEMS) 22 August 1990, see the whole document ---	1,3,6-9 ,11-17
Y	Biological Abstracts, vol. 89, 1990, (Philadelphia, PA, US), G. FRANKEL et al.: "Multi-gene amplification: Simultaneous detection of three virulence genes in diarrheal stool", page 635, abstract no. 72068, & MOL. MICROBIOL. 3(12): 1729-1734. 1989, see abstract ---	1,3,6-9 ,11-17
P,A	Dissertation Abstracts International B, vol. 52, no. 1, July 1991, J.D. MOGOLLON GALVIS: "Evaluation od the use of genetic markers in the study of the epidemiology of Streptococcus suis", page 102, see abstract ---	1
P,X	Infection and Immunity, vol. 59, no. 9, September 1991, U. VECHT et al.: "Identification of two proteins associated with virulence of Streptococcuc suis type 2", pages 3156-3162, see the whole document ---	2-10
Y	-----	1,11-22